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Role of transglutaminase 2 in brain ageing

Ruolo della transglutaminasi 2 nell'invecchiamento del tessuto nervoso

Candidate: Barbara D'Orio

Tutor: Prof.ssa Sandra Moreno

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Contents

Abstract

Transglutaminases (TGs) are a nine members protein family, among which tissue transglutaminase (TG2) is unique for its multiple enzyme activities, ubiquitous expression, cellular localizations and physiological roles. Primary and best studied TG2 function is the Ca^{+2}-dependent transamidating activity, which can result in protein cross-linking between a glutamine and a lysine residue (Iismaa et al., 2009). It is found throughout the body and highly expressed in many tissue, including the nervous one (Thomazy & Fesus, 1989; Chen & Mehta, 1999; Fesus & Piacentini, 2002; Siegel & Khosla, 2007). Within the brain, the enzyme is present in many regions including frontal and temporal cortex, hippocampus and cerebellum (Appelt et al., 1996; Johnson et al., 1997; Kim et al., 1999; Lesort et al., 1999, 2000; Andringa et al., 2004; Wilhelmus et al., 2008). It is primarily neuronal, but can be found in glia as well (Wilhelmus et al., 2008). Originally regarded as a cytosolic protein, TG2 was later found in other cell compartments, including the nucleus, mitochondria, endoplasmic reticulum, as well as in the extracellular space (Lesort et al., 1998; Piacentini et al., 2002; Verderio et al. 2003; Rodolfo et al., 2004; Mishra et al., 2006; Zemskov et al. 2006; Malorni et al., 2009; Szegezdi et al., 2009; Piacentini et al., 2011, Caccamo et al., 2012; Gundemir et al., 2012; Piacentini et al., 2014). Given the ubiquitous expression and vast array of enzymatic and non-enzymatic TG2 activities, it is not surprising that this protein is intimately involved in the regulation of numerous functions, including cell survival and apoptosis (Fesus & Szondy, 2005; Mehta et al., 2006; Verma & Mehta, 2007), autophagy (Mastroberardino & Piacentini, 2010), inflammation (Caccamo et al., 2005b, c; Takano et al., 2010) and oxidative stress processes (Fesus & Szondy 2005; Caccamo et al., 2012). The observed increase of mRNA levels during normal aging and TG2 role in apoptosis, autophagy, ROS imbalance e inflammation create a direct link with the ageing process. Thus it is certain that there are grounds for TG2 to become a new therapeutic target in many age-related diseases in which TG2 contribution has been long described: for instance, cardiovascular disease (Sane et al., 2007), cancer pathology (Budillon et al., 2013), hepatic disease (Nardacci et al., 2003) and age-related neurodegenerative disease, including HD, PD, AD (Jeitner et al., 2009).

Given its involvement in neural cell homeostatic pathways, my PhD project aimed at characterizing the TG2 *in vivo* contribution to mechanisms such as autophagy and antioxidant defense, and how these processes are modulated in

the different brain areas and during normal ageing. This was achieved by studying a knockout mouse model, generated by De Laurenzi & Melino in 2001, carrying a homologous recombination to replace the region of the TG2 gene encoding its catalytic site with the neomycin resistance gene.

The main results obtained from the research and described in this thesis can be summarized as follows:

1. Our data provided *in vivo* evidence of a negative modulatory role of TG2 in the induction phase of autophagy, so far only supported by *in vitro* literature (Akar et al., 2007; Ozpolat et al., 2007; Luciani et al., 2010) Noteworthy, induction of autophagy in $TG2^{-/-}$ mouse brain appears not to be followed by successful completion of the process. This statement is mainly supported by electron microscopic findings concerning (i) accumulation in $TG2^{-/-}$ mouse brain of mitochondria/lipofuscin aggregates, index of decreased lysosomal degradation, (ii) presence of intracellular "myelin figures", reminiscent of residual autophagic bodies, and (iii) deranged Golgi apparatus, sign of lysosomal disturbed biogenesis.
2. Our ultrastructural results highlighted profound alterations of the mitochondrial compartment subsequent to TG2 ablation, consistent with the role of the enzyme in stabilizing complex I and II of the electron respiratory chain (Malorni et al., 2009). Mitochondrial impairment likely leads to superoxide anion leakage, whose accumulation appear aggravated by the decreased expression of its major scavenger SOD2.
3. Our data pointed out that there are brain region-based differences in coping with TG2 ablation. Indeed, neocortex and, partly, the hippocampal formation, regions where ROS detoxifying enzymes are downregulated, are oxidative stress prone areas. By contrast, cerebellar area, long known to be a more protected region of the brain in many neurodegenerative diseases, displays less damaged mitochondria in $TG2^{-/-}$ mice. This could relate to an efficient activation of the antioxidant systems – indeed we detected higher expression of H_2O_2 metabolizing enzymes CAT and GPx 1/2- and/or to a higher autophagic flux.
4. Response to cellular stress caused by TG2 ablation apparently involves peroxisomes, which are biogenetically induced, possibly to cope with mitochondrial deficiency, as it occurs in other pathophysiological situations.
5. Our data clearly show that any attempt by the cell to counteract cell injury consequent to TG2 ablation, is subject to progressive loss of efficiency when

senescence ensues. When comparatively analyzing results from 12- and 24-month-old mice, a worsening of antioxidant defences and autophagic efficiency, associated with ultrastructural damage is detected, even in regions (cerebellum) more resistant to insult.

Our study, by providing novel insights into previously suggested TG2 roles in the brain, and especially by highlighting new functions of the protein in the complex organ, opens the way to future mechanistic investigations, which will hopefully lead to therapeutic applications.

Section I:
Introduction and Objectives

Chapter 1

Tissue transglutaminase

1.1 Tissue transglutaminase structure and enzymatic activity

Transglutaminases (TGs) are a nine members protein family, among which tissue transglutaminase (TG2) is unique for its multiple enzyme activities, ubiquitous expression, cellular localizations and physiological roles.

TG2 is an 80-kDa protein that consists of four structurally distinct domains: an NH_2-terminal β-sandwich, an α/β catalytic core, and two COOH-terminal β-barrel domains (Fig. 1.1.2) (Piacentini et al., 2002). In eukaryotic cells, TG2 is regulated by reversible conformational changes that include Ca^{2+}-dependent activation, which shifts TG2 to the "open" conformation thereby unmasking the catalytic core, and inhibition by GTP, GDP, and ATP, which constrains it in the "closed" conformation (*Fig.1.1.1*) (Nurminskaya & Belkin, 2012; Altuntas et al., 2014). Although recent studies suggested that transamidating activity of TG2 inside and outside the cells is suppressed *in vivo* in the absence of mechanical or chemical stresses (Siegel et al., 2008), it is likely that precise regulation of the enzyme's activity involves other important mechanisms, including binding of Ca^{2+} ions to non-canonical sites (Kiraly et al., 2009), reversible

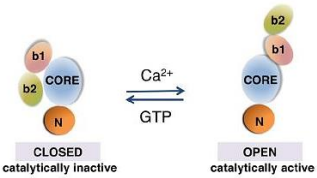

Fig. 1.1.1 Ca^{2+} and guanine nucleotide binding inversely regulate the transamidating TG2 activity (Altuntas et al., 2014)

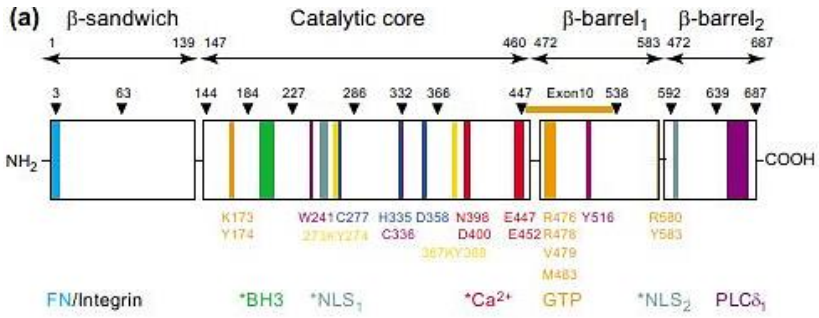

Fig. 1.1.2 Domain structure of TG2 The site of transamidating activity is composed of the catalytic triad of cysteine proteases: cysteine 277 (C277), histidine 335 (H335) and aspartate 358 (D358), which are the critical residues for the enzymatic activity (Fesus & Piacentini, 2002)

reduction/oxidation *via* formation of intramolecular disulfide bonds (Stamnaes et al., 2010), and NO-mediated nitrosylation (Lai et al., 2001).

Primary and best studied TG2 function is the transamidating activity which can result in protein cross-linking - a glutamine residue is cross-linked via an N^{ε}-(γ-glutamyl)lysine isopeptide bond to a lysine residue -, amine incorporation, acylation, esterification and hydrolysis, depending on the substrate (Iismaa et al., 2009).

Besides covalent modification of proteins, TG2 possesses several other enzymatic functions, like for example GTPase activity (Chen & Mehta, 1999; Grosso & Mouradian 2012). TG2 can actually hydrolyze both ATP and GTP into ADP and GDP, respectively, and inorganic phosphate (Achyuthan & Greenberg, 1987; Takeuchi, et al., 1992; Lesort, et al., 2000; Martin, et al., 2006). It is well documented that through this GTPase activity TG2 can act as a G-protein signal transduction molecule *(Fig.1.1.3)* (Nakaoka, et al., 1994; Chen & Mehta, 1999; Lesort, et al., 2000).

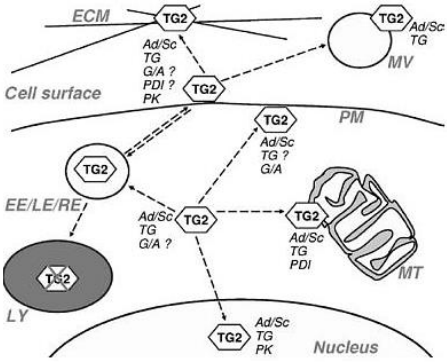

Fig. 1.1.3 Enzymatic and non-enzymatic TG2 activities in diverse cellular compartments. The adapter/scaffolding function of TG2 (Ad/Sc) and its transglutaminase (TG), GTPase/ATPase (G/A), protein disulfide isomerase (PDI), and protein kinase (PK) enzymatic activities are shown for the protein localized in the cytoplasm, underneath the plasma membrane (PM), in the nucleus, in mitochondria (MT), in early/late/recycling endosomes (E/L/RE) and lysosomes (LY), and on the cell surface, in the ECM, and in extracellular microvesicles (MV) (Nurminskaya et al., 2012).

Moreover, TG2 was found to display protein disulfide isomerase (PDI) activity *in vitro* (Hasegawa et al., 2003) and *in vivo* (Mastroberardino et al., 2006; Malorni et al., 2009). More recently, and even more surprisingly, TG2 was suggested to act as a serine/threonine protein kinase *(Fig.1.1.3)* (Mishra & Murphy, 2004; Mishra et al., 2007).

The vast array of TG2 functional activities in the cell is not limited to its enzymatic functions, in fact it has recently emerged an adapter/scaffolding

function of TG2, which is independent of its enzyme activity, and appears to regulate extracellular matrix remodeling, cell adhesion, survival, growth, migration, and differentiation, due to modulation of several signaling pathways *(Fig.1.1.3)* (Belkin, 2011; Wang & Griffin, 2012; Nurminskaya et al., 2012).

1.2 Tissue transglutaminase localization and expression regulation

TG2 is highly expressed in kidney, colon, liver, heart, lung, spleen and nervous tissue (Thomazy & Fesus, 1989; Chen & Mehta, 1999; Fesus & Piacentini, 2002; Siegel & Khosla, 2007), and found throughout the body due to its constitutive expression in endothelial cells, smooth muscle cells, and fibroblasts.

Remarkably, the expression of this protein is regulated on many levels and can be strikingly and acutely induced in response to a number of unrelated stressors, including injury, inflammation, and neoplastic transformation. Oxidants, hypoxia, oncogenes, cytokines, and growth factors all potently regulate TG2 in different cell types (Ientile et al., 2007) *(Fig.1.2.1)*. In agreement, a number of transcription factor-binding sites have been identified in the promoter region of the respective mouse gene TGM2 (Lu et al., 1995; Nagy et al., 1996; Ritter & Davies, 1998). Indeed, one important aspect of TG2 regulation is the frequent involvement of inflammatory mediators. There is in fact, a response element-binding site for transforming growth factor (TGF) family proteins in the mouse TGM2 promoter, although whether binding of this element leads to up-regulation or down-regulation of TG2 is cell type specific (Ritter & Davies, 1998). This becomes highly relevant in light of the important contribution of inflammation in neurodegenerative diseases through which TG2 can potentially be upregulated in these disorders. Although interferon (IFN)-stimulated response elements in the

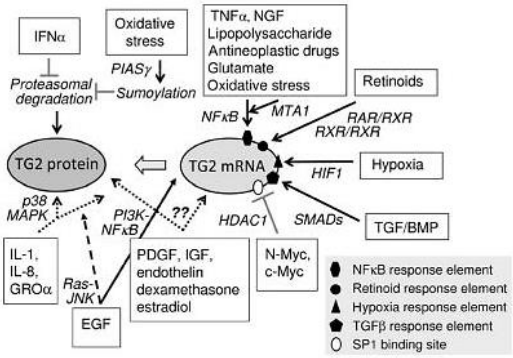

Fig. 1.2.1 Regulation of TG2 expression (Nurminskaya et al., 2012)

TGM2 promoter are not characterized, IFNα2b was shown to modestly increase the transcription of the gene in diffent cancer cells lines (Giandomenico et al., 1997; Esposito et al., 2003), as for interleukin1 and interleukin-8 increase TG2 expression and activity in osteoarthritic chondrocytes (Jhonson et al., 2001; Merz et al., 2003). Activation of the NFκB signaling pathway was reported to acutely induce TG2 mRNA expression in hepatocytes in response to chemical injury (Mirza et al., 1997), as well as interleukin-6 and TNFα (Kuncio et al., 1998). A large and growing body of work indicates that excessive activation of the NFκB pathway might be particularly important for inducing increased levels of TG2 expression during inflammatory responses and in many types of tumor cells (Mehta et al., 2010). Retinoids were historically the first factors found to markedly induce the acute upregulation of TGM2 gene transcription in different cells type (Chiocca et al., 1989; Piacentini et al., 1992a, b; Vollberg et al., 1992). Accordingly, ~1.7kb upstream of the transcription start site, the TGM2 promoter was found to contain a versatile tripartite retinoid response element which is activated by either retinoic acid receptor-retinoid X receptor (RAR/RXR) heterodimers or RXR omodimers (Nagy et al., 1996).

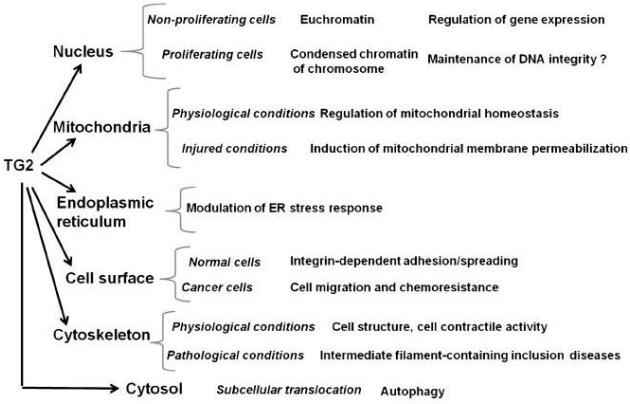

Fig. 1.2.2 The multifunctional enzyme TG2 is found in various sub-cellular compartments in which its activity is regulated by interaction with diverse factors depending on its location (Piacentini et al., 2014)

Noteworthy, the diverse function(s) of TG2 are closely related to its cellular or subcellular location under different physiological or pathological conditions (Wang & Griffin, 2012). Originally regarded as a cytosolic protein, TG2 was later found in other cell compartments, including the nucleus, mitochondria, endoplasmic reticulum, as well as in the extracellular space *(Fig.1.2.2)* (Lesort et al., 1998; Piacentini et al., 2002; Verderio et al.

2003; Rodolfo et al., 2004; Mishra et al., 2006; Zemskov et al. 2006; Malorni et al., 2009; Szegezdi et al., 2009; Piacentini et al., 2011, Caccamo et al., 2012; Gundemir et al., 2012; Piacentini et al., 2014).

1.2.1 Functions of mitochondrial TG2

The initial evidence that TG2 acts as an important regulator of energy metabolism and mitochondrial functions derives from observations on $TG^{-/-}$ mouse heart, which is more susceptible than wild-type (WT), to ischemia-reperfusion injury (Szondy et al., 2006; Sarang et al., 2009). The deletion of TGM2 in mice causes significant dysregulation of the respiratory complexes I and II, reduction of ATP production, increased ATP/ADP carrier activity and mitochondrial membrane potential, and impairment of ATP synthase reverse activity and Bax recruitment (Malorni et al., 2009). Indeed, Bax, a pro-apoptotic protein and TG2-binding partner, appears to serve as the major target of TG2-induced cross-linking during apoptosis (Rodolfo et al., 2004). Accordingly, TG2 overexpression in neural cells results in a much more rapid execution of death program, accompanied by clustering of mitochondria, reduced cristae, and condensed matrix (Piacentini et al., 2002).

Recent work revealed a major role of TG2 in mitochondrial physiology and energy metabolism also acting as a PDI (Mastroberardino et al., 2006; Malorni et al., 2009; Sarang et al., 2009) and several studies identified the mitochondrial substrates of TG2 transamidating activity *in situ* (Sarang et al., 2009; Park et al., 2010). While very few of such TG2-mediated modifications take place in unaffected healthy tissues, they are likely to be involved in the pathogenesis of "mitochondrial diseases", including cardiovascular ischemia/reperfusion injury and neurodegenerative disorders.

1.3 Tissue transglutaminase distribution in the nervous tissue

TG2 is a normal component of central (CNS) and peripheral (PNS) nervous systems (Selkoe et al., 1982; Gilad & Varon, 1985a, b; Ohashi et al., 1995) and its activity is developmentally regulated (Maccioni & Seeds, 1986; Perry & Haynes, 1993). In rat brain and spinal cord, TG activity is highest in the late fetal stages when axonal outgrowth is occurring (Chakraborty et al., 1987; Perry & Haynes, 1993).

Within the brain, the enzyme is present in many regions including frontal and temporal cortex, hippocampus, substantia nigra and cerebellum (Appelt et al.,

1996; Johnson et al., 1997; Kim et al., 1999; Lesort et al., 1999, 2000; Andringa et al., 2004; Wilhelmus et al., 2008). It is primarily neuronal, but can be found in glia as well (Wilhelmus et al., 2008). In neurons, TG2 is located in both the cell body, with dense perinuclear concentration, and at the distal tips of neuritic processes (Lesort et al., 1999).

Nuclear localization of TG2 also has been reported (Singh et al., 1995; Lesort et al., 1998) although levels in unstimulated cells are low. However, when intracellular Ca^{2+} levels are increased, TG2 can translocate into the nucleus where it acts as a transamidating enzyme. In addition, it has been demonstrated that TG activity increases during regeneration of the sciatic nerve in rats (Chakraborty et al., 1987).

In the nervous system, TG2 has been postulated to play a role in synaptic plasticity, release of neurotransmitters (Pastuszko et al., 1986), long-term potentiation (Friedrich et al., 1991), axonal regeneration (Chakraborty et al., 1987), and neuronal differentiation. Indeed, TG2 is both necessary and sufficient for neuronal differentiation of human neuroblastoma SH-SY5Y cells and the neurodifferentiation resulting from increased expression of TG2 is dependent upon its transamidating activity (Mahoney et al., 2000; Tucholski et al., 2001).

In humans, increases in TG2 mRNA levels have been observed during normal aging (Lu et al., 2004). Moreover, enhanced expression of TG2 has been documented in some chronic neuropathological conditions such as Alzheimer's disease (Johnson et al., 1997; Kim et al., 1999), Huntington's disease (Karpuj et al., 1999; Lesort et al., 1999), Parkinson's disease (Junn et al., 2003; Andringa et al., 2004) and supranuclear palsy (Zemaitaitis et al., 2003). TG2 expression and/or activity is also increased in animal models of acute CNS damage including spinal cord injury (Festoff et al., 2002), traumatic brain injury (Tolentino et al., 2002) and ischemia (Tolentino et al., 2004). Because of the well-documented increase in TG activity and TG2 mRNA and protein levels during cell death processes in different models, it has been hypothesized that TG2 facilitates neuronal cell death in neurodegenerative diseases, as well as in response to CNS injury. This hypothesis is supported by studies in which crossing Huntington's disease transgenic mice with TG2 knockout mice results in a significantly delayed onset of motor dysfunction and prolonged survival of diseased mice (Mastroberardino et al., 2002; Bailey & Johnson, 2005).

Furthermore, Tucholski and coll. (2006) provided the first *in vivo* evidence that TG2 facilitates kainic acid-induced cell death. Since increased expression of TG2 greatly enhances excitotoxic neuronal cell death, the development of TG inhibitors may be a promising strategy for the treatment of neurological conditions such as Alzheimer's disease, Huntington's disease and traumatic brain injury in which excitotoxicity likely plays a contributing role to the pathogenic processes (Lynch & Dawson, 1994; Jakel & Maragos, 2000; Hynd et al., 2004).

1.4 Tissue transglutaminase role in cell processes

Given the ubiquitous expression and vast array of enzymatic and non-enzymatic TG2 activities, it is not surprising that this protein is intimately involved in the regulation of numerous functions, including cell adhesion, migration, growth and differentiation, exocytosis, ECM assembly and turnover (Nurminskaya & Belkin 2012).

In the past decade, numerous studies focused on the role of TG2 in cell survival and apoptosis (Fesus & Szondy, 2005; Mehta et al., 2006; Verma & Mehta, 2007). Apoptosis is a fundamental process, playing a critical role in normal tissue homeostasis, as well as in disease. TG2 gene was identified among those closely related to the final execution of the apoptotic process (Fesus, 1992). Today, the dual role of TG2 acting either as a facilitator or

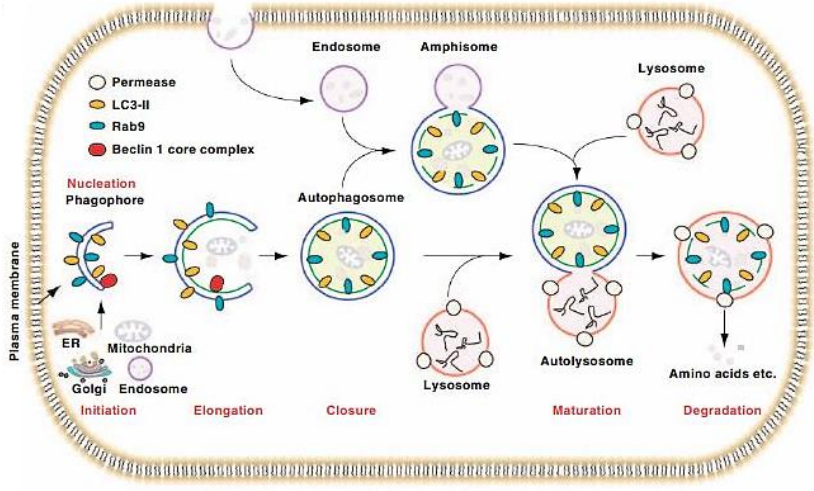

Fig. 1.4.1 Stages of autophagic processes (Kang et al., 2011)

attenuator of the apoptotic process is widely acknowledged (Fesus & Szondy, 2005). The current general concept implies that TG2 sensitizes cells to apoptosis when its transamidating activity is turned on; in contrast, it is protective when its transamidating activity is dormant (Antonyak et al., 2001; Tucholski & Johnson, 2002; Milakovic et al., 2004).

In addition to its role in apoptosis, TG2 is involved in the regulation of autophagy (Mastroberardino & Piacentini, 2010). The latter is a complex process divided into six principal steps, thoroughly characterized in yeast cells: initiation, nucleation, elongation, closure, maturation and degradation or extrusion. The initiation is typically inhibited by mTOR and leads to phagophore formation, mainly from endoplasmic reticulum. Then follows the nucleation, that depends on Beclin 1-Vps34-Vps15 core complexes and other proteins, including AMBRA1. Elongation of the phagophore is mediated by two ubiquitin-like conjugation systems that together promote ATG16L assembly and LC3 processing. Maturation is promoted by different factors, i.a., LC3, Beclin 1, the lysosomal membrane proteins LAMP-1 and LAMP-2, and lysosomal pH. Autophagosomes then fuse with lysosomes converting them into autolysosomes. The content of the autophagosomes is therefore degraded by lysosomal hydrolases and the metabolites generated as a result of autophagy are reused either as sources of energy or building blocks for the synthesis of new macromolecules (Kang et al., 2011) *(Fig.1.4.1).*

In the absence of TG2, cell stressors cause an enhanced induction of autophagy, suggesting that TG2 inhibits autophagy (Akar et al., 2007), probably as a downstream effect mediated by protein kinase C-δ (PKC δ) that

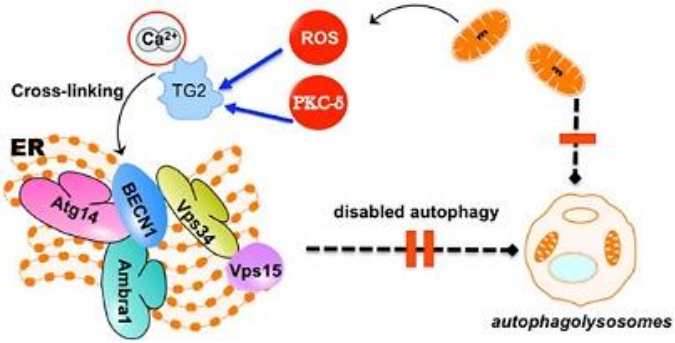

Fig. 1.4.2 TG2 activation leads to BECN1 crosslinking and displaces BECN1-interactome away from the endoplasmic reticulum (ER). This mislocalization inhibits autophagosome formation and disables autophagy (modified from Villella et al., 2013)

constitutively suppresses autophagy through TG2 induction (Ozpolat et al., 2007). One mechanism for this is the ability of TG2 to cross-link beclin1, which is a key player in autophagosome formation, and sequester it in aggresomes; thus, blocking TG2 activity increases autophagy (Luciani et al., 2010). However, TG2-/- mesenchymal embryonic fibroblasts (MEF) have impaired maturation of pre-autophagic vesicles to autophagolysosomes, suggesting that TG2 is needed for vescicle maturation (D'Eletto et al., 2009). While further studies are required to determine the precise role of TG2 in autophagy, one possible explanation is that TG2 inhibits the early-stage induction of autophagy, but is necessary for the completion of this process *(Fig1.4.2).*

Intracellular reactive oxygen species (ROS) accumulation has a number of direct and indirect consequences on cell signaling pathways, ultimately leading to apoptosis or necrosis. In the last decade, numerous studies investigating the involvement of TG2 in oxidative stress-induced cell death, have reported conflicting data showing that TG2 exhibits a different behavior

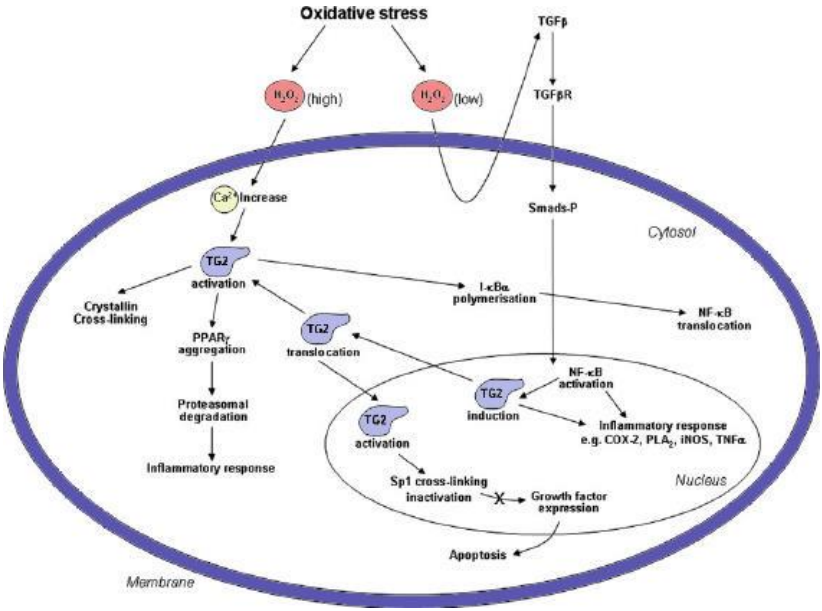

Fig. 1.4.3 Signaling pathways involving TG2 activation in cell response to oxidative stress (modified from Caccamo et al., 2012)

in cell response to injury/stress, acting either as a facilitator or attenuator of the apoptotic process *(Fig 1.4.3)* (Fesus & Szondy 2005).

Recent investigations on cardiac myocyte apoptosis under oxidative stress, a condition that can be observed in ischemic conditions, show that the increase in the expression of apoptotic markers, is positively correlated with TG2 up-regulation in H_2O_2-treated cardiomyocytes (Song et al., 2011). These data suggest a strong association between oxidative stress and TG2 up-regulation. This latter in turn may result in cell survival or apoptosis, depending on the cell type, the kind of stressor and the duration of insult (Antonyak et al., 2001, 2003; Tucholski & Johnson 2002).

Moreover, several ER stress-causing agents increased TG2 in situ transamidating reaction, conversely the ER stress-induced protein aggregation was reduced by treatment with a TG2 inhibitor. These results demonstrate that unfolded or misfolded proteins produced by oxidative stress activate TG2, leading to the accumulation of protein aggregates (Lee et al., 2014).

Further, TG2 was demonstrated as a necessary component of oxidative stress-induced death machinery in cortical neurons. As oxidative stress has been implicated in most acute and chronic neuropathological conditions (Caccamo et al., 2004, 2010; Ruan & Johnson, 2007), these findings suggest that even in diseases characterized by proteotoxicity, TG2 major role may not be limited to crosslinking but also include cell death signaling (Basso et al., 2012).

Several experimental studies have demonstrated an age-related sensitivity of astroglial cells to oxidative stress, which makes neurons more susceptible to injury (Wang et al., 2006; Jou, 2008). The exposure of astrocytes primary cultures to excitotoxic glutamate concentrations resulted in TG2 up-regulation associated with oxidative stress. The pre-incubation with antioxidants was able to recover redox basal conditions and decrease TG2 levels (Campisi et al., 2004; Caccamo et al., 2005a). This latter effect was achieved by the reduction of glutamate-induced NF-κB activation, given that experiments with a specific NF-κB inhibitor demonstrated the NF-κB involvement in TG2 up-regulation (Caccamo et al., 2005b, c). Further evidence of the involvement of NF-κB activation in TG2 up-regulation associated with oxidative stress has been provided by experiments in cultured

rat hippocampal astrocytes activated by lipopolysaccharide (LPS), which is generally used for a stimulant of iNOS induction. Both TG2 and iNOS up-regulation induced by LPS stimulation were suppressed by an inhibitor for NF-κB activation, and an antioxidant in a dose dependent manner (Takano et al., 2010).

1.5 Mouse models of genetically altered expression of tissue transglutaminase

TG2 is implicated in a number of diseases including many neurodegenerative disorders. Genetically altered mice, either knockout for or overexpressing TG2, have been generated in order to further study its role *in vivo*. Since TG2 is the predominant form of the family in the mouse forebrain, these studies are expected to be informative about the function of this protein in the brain (Ruan & Johnson, 2007). $TG2^{-/-}$ mice were generated independently by two different groups. Nanda et al. (2001) used the Cre/LoxP site-specific recombinase system to delete the catalytic core domain, causing a frameshift that blocks downstream transcription of TG2. The second line of deficient mice was generated by De Laurenzi and Melino (2001), by homologous recombination to replace the region of the TG2 gene encoding its catalytic site with the neomycin resistance gene. Mice from both lines are viable, grow to normal size, have no reproductive abnormalities, and show no apoptosis impairment. However, they do demonstrate decreased fibroblast adhesion in culture, increased susceptibility of thymocytes to dexamethasone-induced cell death, and a smaller thymus than WT mice. Additionally, $TG2^{-/-}$ mice show impaired clearance of apoptotic cells leading to a "lupus-like" autoimmune phenotype with B cell hyperplasia and production of anti-nuclear antibodies (Szondy et al., 2003; Siegel & Khosla, 2007). These mice also develop a diabetes-type syndrome with impaired insulin secretion following a glucose load and increased sensitivity to insulin in peripheral tissues (Bernassola et al., 2002; Griffin et al., 2002), and are more susceptible to hepatic insults (Nardacci et al., 2003; Sarang, et al., 2005). Further, mitochondrial abnormalities, associated with impaired ATP production (Szondy et al., 2006), decreased mitochondrial complex I activity, and increased sensitivity to methamphetamine and the complex II inhibitor 3-nitropropionic acid (Battaglia et al., 2007) are described in $TG2^{-/-}$ mice. Additionally, they exhibit heart function deficits at baseline and impaired motor performance on the rotarod, which are attributed to energy depletion

in skeletal and cardiac muscles resulting from mitochondrial abnormalities (Szondy et al., 2006). TG2 transgenic mice using different promoters are also informative. For example, overexpressing TG2 in the heart using the mouse α-myosin heavy chain promoter results in cardiac hypertrophy and interstitial fibrosis (Small et al., 1999). Another TG2 transgenic mouse line generated using the murine prion promoter to overexpress TG2 in the brain, with a smaller amount in the heart, when challenged with kainic acid, displays exacerbated hippocampal damage and increased seizure activity relative to WT mice (Tucholski et al., 2006).

1.6 Tissue transglutaminase and the ageing connection

Senescence is characterized by a progressive impairment of mechanisms that protect cells from multifactorial injuries: DNA damage, accumulation of toxic metabolites (e.g., ROS), protein aggregates, dysregulation of inflammatory response and mitochondrial dysfunction. Among these processes, increasing evidence suggests an important role for programmed cell death (PCD) pathways, which are critical to tissue homeostasis. During aging, accumulated cellular damage and inefficient systemic signaling can cause too little cell death (hyperproliferation and cancer), or too much cell death (tissue atrophy and ectopic cell death), thereby limiting tissue function and life span (Shen & Tower, 2009).

Moreover, it is well recognized that cellular homeostasis relies on autophagy, which plays a key role in removing damaged organelles and other cell components. During aging, the efficiency of autophagic degradation declines and intracellular waste products accumulate. In *Caenorhabditis elegans*, there is clear evidence that lifespan is linked to the capacity to regulate autophagy. Recent studies have revealed that the same signaling factors regulate both aging and autophagocytosis, thus highlighting the relevance of this process in aging and age-related degenerative diseases (Salminen & Kaarniranta, 2009). Mitochondria play a central role in physiological and pathological ageing and increased apoptosis and dysfunctional autophagy may be related to their damage. This in turn depends on mitochondrial membrane potential and cellular redox status. Therefore, age-associated cell loss may be due to mitochondrial-triggered apoptosis caused by imbalance of pro-oxidant *vs.* antioxidant pathways, or by increased activation of

mitochondrial permeability transition pores (Pollack et al., 2002). Alterations in energy metabolism can also be connected to the lower mitochondrial functionality. To date, the free radical and mitochondrial theories seem to be the two most prominent theories on aging and have survived the test of time. Such theories claim that oxidative stress within mitochondria can lead to a vicious cycle in which damaged mitochondria produce increased amounts of ROS, leading in turn to progressive augmentation in damage (Romano et al., 2010).

As above mentioned, TG2 is a multifunctional protein involved in different cellular processes, particularly those involved in elderly age. The observed increase of mRNA levels during normal aging and TG2 role in apoptosis, autophagy, ROS imbalance e inflammation create a direct link with the ageing process. How tight is this link and how TG2 modulation may modify/improve the physiopathological aging, is yet to be understood.

It is certain though that there are grounds for TG2 to become a new therapeutic target in many age-related diseases: cardiovascular disease for instance, seen the role in cardiovascular biology, including contributing to the development of hypertension, influencing the progression of atherosclerosis, regulating vascular permeability and angiogenesis and contributing to myocardial signaling, contractile activity and ischemia/reperfusion injury (Sane et al., 2007); cancer pathology, TG2 has indeed emerged as a putative gene involved in tumor cell drug resistance and evasion of apoptosis (Budillon et al., 2013); hepatic disease, since TG2 plays a protective role in the liver injury by favoring tissue stability and repair (Nardacci et al., 2003).

Importantly, application of knowledge about function and modulation of TG2 may impact therapeutic approaches against neurodegenerative diseases, based on the physical interaction of this enzyme with the main protein constituents of morphological hallmarks of HD, PD, AD (Jeitner et al., 2009). Indeed, huntingtin, amyloid β, α-synuclein are reported as substrates of TG2 (Wang et al., 2008; Schmid et al., 2009; McConoughey et al., 2010; Mastroberardino and Mauro Piacentini, 2010; Wilhelmus et al., 2011) and neuronal damage, either due to environmental insults or to genetic predisposition, leads to conditions favorable to TG2 activation.

Chapter 2

Objectives

TG2 is implicated in a number of age-related neurodegenerative disorders including Huntington's (HD), Alzheimer's (AD), and Parkinson's diseases (PD), basing on the evidence that most of these pathologies include the formation of insoluble aggregates, possibly produced by TG2 cross-linking activity, the latter promoted by low GTP and high calcium levels, a condition frequently met in in neurodegenerative diseases. Moreover, ROS accumulation, a well-known occurrence in these diseases, upregulates TG2 expression and activity. The link between TG2 and neurodegenerative diseases, is strengthened by its role in survival/death pathway, considering the dysregulated autophagy in the pathogenesis of these disorders (Grosso & Mouradian, 2012).

Given its involvement in neural cell homeostatic pathways, the present investigation aimed at characterizing the *in vivo* role of TG2 in the brain during normal ageing. This was achieved by studying a knockout mouse model, generated by De Laurenzi & Melino in 2001, carrying a homologous recombination to replace the region of the TG2 gene encoding its catalytic site with the neomycin resistance gene. We focused on the contribution of TG2 to mechanisms such as autophagy and antioxidant defense, analysing how these processes are modulated in the different brain areas, during normal ageing.

To address our issues, we used morphological approaches, namely immunohistochemistry, immunofluorescence, and electron microscopy, which are especially suitable to the study of the nervous tissue, for its highly complex organization and heterogeneous composition, but we also quantitatively assessed protein expression by means of Western blotting analysis. We decided to conduct our investigations at two time-points, choosing adult mice of 12 months old, and old mice aging 24 months.

The effect of TG2 deletion has been evaluated primarily on adult mouse liver, an organ displaying high levels of TG2 expression and particularly significant for age-related studies. This initial analysis gave us some first clues on the induction of autophagy following TG2 ablation, on the mitochondrial suffering and on some metabolic alterations present in the knockout mice.

We then moved to the main topic of the project, *i.e.*, the brain ageing study, focussing on those regions especially susceptible to neurodegeneration (neocortex, hippocampus) or relatively spared (cerebellum) as a reference area.

To carry out our study on both liver and ageing brain, different autophagy markers (AMBRA1 and Beclin1) and antioxidant enzymes (SOD1, SOD2, GPx 1/2 and catalase) were evaluated by immunoblotting and immunohistochemical analysis. Ultrastructural morphology was assessed by transmission electron microscopy in $TG2^{-/-}$ animals, as compared to WT, analysing the features of different neuronal populations, including neocortical and hippocampal pyramidal neurons, and Purkinje cells of the cerebellar cortex. Moreover, in the context of the brain study, we also assessed astroglial activation by means of immunofluorescence techniques.

Overall, the present study provides information on the contribution *in vivo* of TG2 to mechanisms such as autophagy and antioxidant defense, and how these processes are modulated in the different brain areas and during normal ageing. Getting this basic information will allow in the future to deepen specific mechanisms highlighted by this study and to move to translational studies focused on pathological conditions, in order to test TG2 as a novel therapeutic target.

Section II:

Results and Discussion

Chapter 3

Effects of TG2 deletion in the liver

The effects of TG2 ablation were evaluated primarily on the liver, an organ presenting high levels of TG2 expression and particularly significant for age-related studies.

3.1 Immunoblotting analysis

Immunoblotting data on 12-month-old mouse liver provided the first information about TG2 regulation of the autophagic pathway and antioxidant defences.

The autophagy markers Beclin1 and AMBRA1, which form a complex important for the localization of autophagic proteins to a pre-autophagosomal structure, show higher levels in TG2$^{-/-}$ mice in respect to their WT counterparts *(Fig.3.1.1)*. While in line with previous *in vitro* studies, demonstrating suppression of autophagy by TG2 (Akar et al., 2007; Ozpolat et al., 2007), ours represents the first *in vivo* evidence of such inhibitory properties of TG2. As to the mechanisms underlying this action, it is worth considering that TG2 is able to cross-link Beclin1, sequestering it in aggresomes (Luciani et al., 2010), leading to its clearance. Therefore, the higher abundance of Beclin1 may reflect lower degradation rate, rather than enhanced expression of the protein.

Notwithstanding the induction of the autophagy, whether this is followed by the completion of the pathway itself, is yet to be determined. Interestingly, embryonic fibroblasts from TG2$^{-/-}$ mice have impaired maturation of pre-

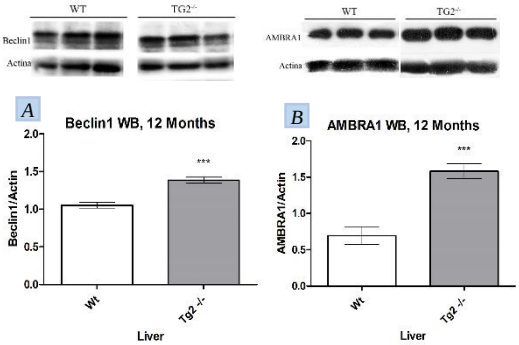

Fig.3.1.1 Densitometric analysis of Beclin1 WB assay (A) and AMBRA1 WB assay (B) of liver from WT and TG2$^{-/-}$ mice, ageing 12 months. * p<0,05; **p<0,01.

autophagic vesicles to autophagolysosomes, suggesting that TG2 is needed for the maturation of autophagic vesicles (D'Eletto et al., 2009).

As markers of antioxidant activity, we chose catalase (CAT), mostly located in the peroxisomes, glutathione peroxidase 1/2 (GPx 1/2), mainly a cytosolic enzyme, superoxide dismutase 1 (SOD1), widely distributed in cell compartments, and SOD2, typically mitochondrial.

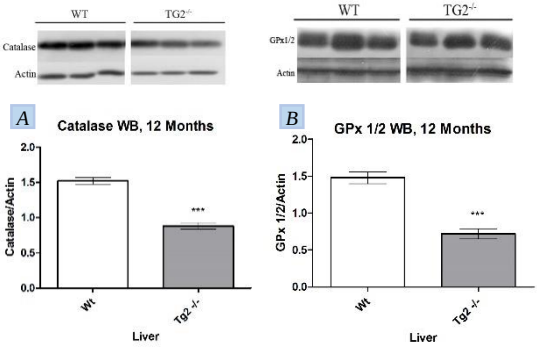

Fig.3.1.2 Densitometric analysis of CAT WB assay (A) and GPx 1/2 WB assay (B) of liver from WT and $TG2^{-/-}$ mice, ageing 12 months. * p<0,05; **p<0,01.

The two H_2O_2-detoxifying enzymes show remarkably lower levels of expression in $TG2^{-/-}$ mice *(Fig.3.1.2)*. Similarly, SOD2, converting toxic superoxide - a byproduct of the mitochondrial electron transport chain - into hydrogen peroxide and diatomic oxygen, appears downregulated *(Fig.3.1.3 B)*. While contributing to the hypothesis that in the absence of TG2 redox status is altered in hepatocytes, this finding also supports mitochondrial impairment. Indeed, deletion of TG2 in cell cultures, cause significant dysregulation of the mitochondrial respiratory complexes I and II, reduction of ATP production, increased ATP/ADP carrier activity and mitochondrial

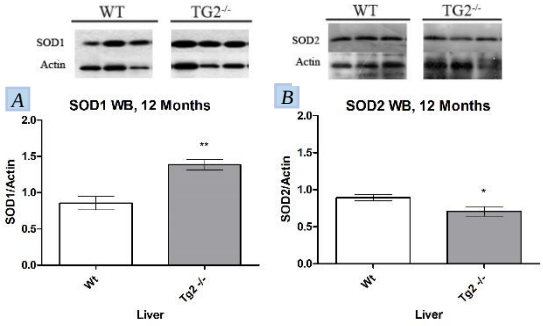

Fig.3.1.3 Densitometric analysis of SOD2 WB assay (A) and SOD1 WB assay (B) of liver from WT and $TG2^{-/-}$ mice, ageing 12 months. * p<0,05; **p<0,01.

membrane potential, and impairment of ATP synthase reverse activity and Bax recruitment (Malorni et al., 2009).

Opposite behavior, in respect to the above antioxidant enzymes, is shown by SOD1, whose levels in $TG2^{-/-}$ mouse liver are abnormally high *(Fig.3.1.3 A)*. This intriguing finding suggests compensatory mechanisms triggered by oxidative stress in different cytosol/peroxisomes/mitochondrial compartments, in an attempt to buffer the high load of ROS production. It should be noted, however, that the increased expression of SOD1 may result in enhanced production of H2O2 not counterbalanced by its removal. Therefore, an even exacerbated oxidative stress condition may ensue in $TG2^{-/-}$ liver. This is consistent with the proposed involvement of TG2 in the maintenance of redox homeostasis, as this enzyme is normally upregulated subsequent to a burst of oxidative stress (Caccamo et al., 2012). It will be relevant to further characterize this relatively unexplored role of TG2, possibly leading to the transcription of a plethora of different genes related to inflammation/ROS pathways, in which CAT, GPx 1/2 and SOD2 may be included.

3.2 Ultrastructural analysis

While the overall morphology and cytoarchitecture of liver tissue from $TG2^{-/-}$ appears comparable to WT, the ultrastructural analysis revealed alterations of some cellular compartments. Accumulation of glycogen and lipid droplets is a common finding in both 12- *(Fig.3.2.1, D, E, F)* and 24- *(Fig.3.2.2, D, E, F)* month-old $TG2^{-/-}$ mice. Explanation for these results may relate to TG2 being a negative regulator of adipogenesis, in agreement with TG2 deficient MEFs, displaying accelerated lipid accumulation due to increased expression of major adipogenic transcription factors, namely peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins alpha(C/EBPα) (Myneni et al., 2015).

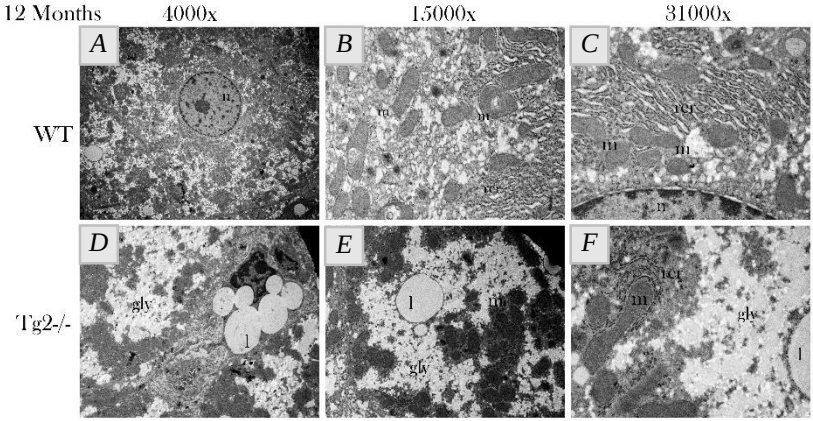

Fig.3.2.1 Ultrastructural liver analysis of 12 months old WT (A, B, C) and $Tg2^{-/-}$ (D, E, F) mice. In the knockouts larger glycogen deposits (gly) and lipid droplets (l) in respect to the WT are observed, while nucleus (n), rough endoplasmic reticulum (rer), and mitochondria (m) appear to be normal.

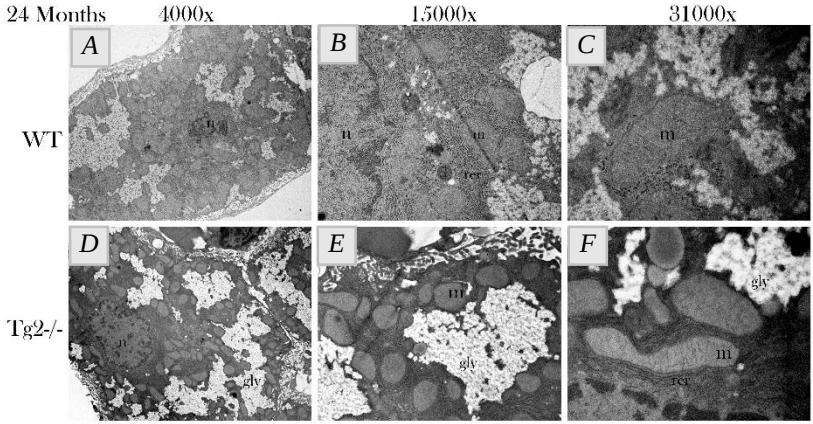

Fig.3.2.2 Ultrastructural liver analysis of 24 months old WT (A, B, C) and $Tg2^{-/-}$ (D, E, F) mice. In the knockouts larger glycogen deposits (gly) in respect to the WT are observed, while nucleus (n), rough endoplasmic reticulum (rer), and mitochondria (m) appear to be normal.

Chapter 4

Effects of TG2 ablation in the ageing brain

The main purpose of my PhD project was to evaluate the effects of TG2 ablation in the ageing brain, an organ in which TG2 is the major isoform of the TGs family, being present in several regions including frontal and temporal cortex, hippocampus, *substantia nigra* and cerebellum (Appelt et al., 1996; Johnson et al., 1997; Kim et al., 1999; Lesort et al., 1999, 2000; Andringa et al., 2004; Wilhelmus et al., 2008). For this investigation, we used a combined molecular/morphological approach, taking advantage of immunoblotting, immunohistochemistry, immunofluorescence and ultrastructural techniques. Analyses were carried out on 12- and 24-month-old $TG2^{-/-}$ and WT mice, focusing on those brain areas especially susceptible to neurodegeneration (neocortex, hippocampus) or relatively spared (cerebellum), for comparison.

4.1 Ultrastructural analysis

Brain morphological analyses provided us with important clues on the cellular alterations consequent to TG2 ablation. These affect fundamental organelles and related cell processes – energy metabolism, protein synthesis and modification, degradation processes.

In the neocortex of $TG2^{-/-}$ mice, at both 12 *(Fig.4.1.1, C)* and 24 months *(Fig.4.1.2, C)*, we can see a greater deposition of finely granular yellow-brown pigment granules, referred to as lipofuscin. These deposits of lipid-containing residues of lysosomal digestion, are considered one of the aging or "wear-and-tear" pigments. Lipofuscin appears to be the product of the oxidation of unsaturated fatty acids and may be symptomatic of membrane damage, or damage to mitochondria and lysosomes. In fact, mitochondria result severely damaged at 12 months *(Fig.4.1.1, D)*, with internal *cristae* shredded in pieces and an inner matrix less electron-dense than in the WT *(Fig.4.1.1, B)*. At 24 months, mitochondria appear even more injured *(Fig.4.1.2, D)*, since appearing just as “ghosts”, where the inner and outer membranes are barely visible. Thus, mitochondrial lesions are associated with lipofuscin formation in the neuronal cell body *(single arrow in Fig.4.1.1 C; 4.1.2, C)*, giving support to the hypothesis that mitochondria are a major

substrate for lipofuscin formation, possibly as a result of incomplete mitophagy.
Remarkably, the bodies we observed by TEM are impressively similar to the ones described by Aliev and coll. (2008) in human AD samples, suggesting that $TG2^{-/-}$ phenotype resembles at least in some aspects this devastating neurodegenerative disease.

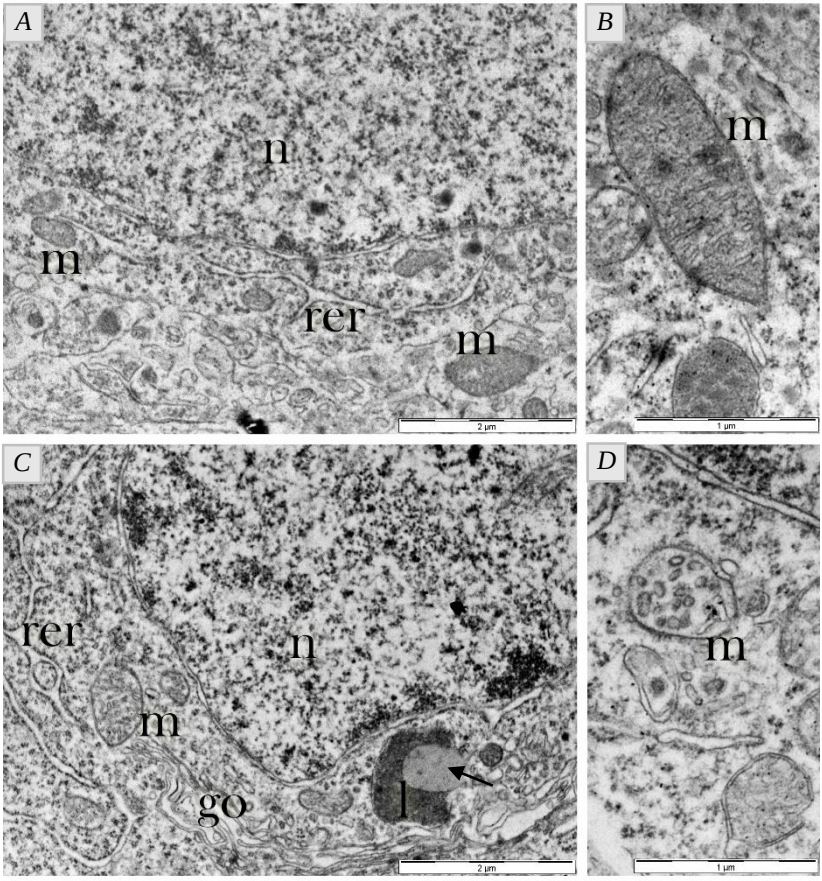

Fig.4.1.1 Ultrastructural analysis of brain neocortex from 12 months old mice. (A) Normal morphology of a WT neuronal cell at 15000x magnification (B) Detail of a normal mitochondria of a WT neuronal cell at 30000x magnification (C) Morphology of a $TG2^{-/-}$ neuronal cell, with lipofuscin deposit (l), altered Golgi apparatus (go), and injured mitochondria (m) at 15000x magnification (D) Detail of an injured mitochondria from a $TG2^{-/-}$ neuronal cell with disrupted internal *cristae* at 30000x. n,nucleus; Rer, rough endoplasmic reticulum

Even the Golgi apparatus of neocortical neurons from knockout mice aging 12 and 24 months *(Fig.4.1.1, C)* appears morphologically altered with disaggregated *cisternae* in contrast to the WT, in which these structures appear correctly arranged in stacks and flattened (*for comparison see Fig.4.1.6 A*). Noteworthy, alterations to this compartment may lead to

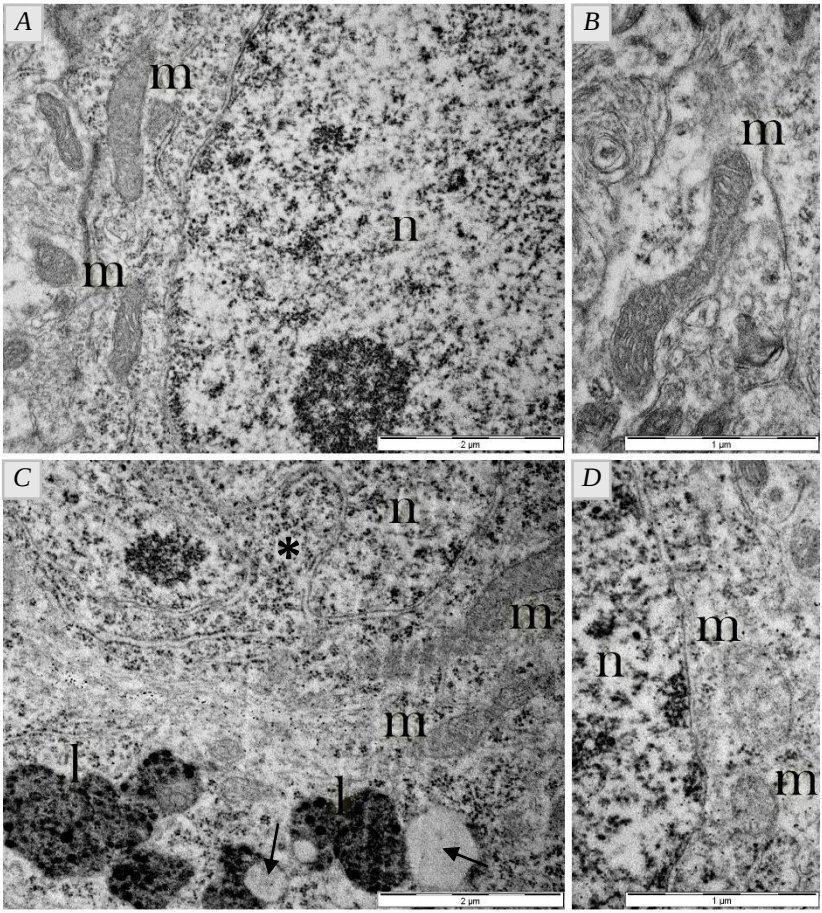

Fig.4.1.2 Ultrastructural analysis of brain neocortex from 24 months old mice. (A) Normal morphology of a WT neuronal cell at 15000x magnification (B) Detail of a normal mitochondria (m) from a WT neuronal cell at 30000x magnification (C) Morphology of a $TG2^{-/-}$ neuronal cell, with lipofuscin deposit (l), nucleus indentation (*) and injured mitochondria at 15000x magnification (D) Detail of the "ghosts" of mitochondria from a $TG2^{-/-}$ neuronal cell 30000x

dysfunctional lysosomes, since their biogenesis and maturation relies on the Golgi complex. This suggests impaired clearance of autophagosomes (thereby including mitochondria-containing ones) and consequent accumulation of undigested material in the form of lipofuscin. Further support to this hypothesis derives from an additional ultrastructural result obtained for both 12- *(Fig.4.1.3)* and 24-month-old $TG2^{-/-}$ mouse neocortex *(Fig.4.1.4)*, that is the presence of the so-called "myelin figures", lipid structures evocating autophagic vacuoles. Overall, the occurrence of autophagy-reminiscent structures, interpreted in the light of the role of TG2 in the maturation of autophagic vesicles (D'Eletto et al., 2009), is a convincing evidence of autophagy progression impairment in $TG2^{-/-}$ neurons. Moreover, nucleus indentation is a frequent TEM finding in $TG2^{-/-}$ samples

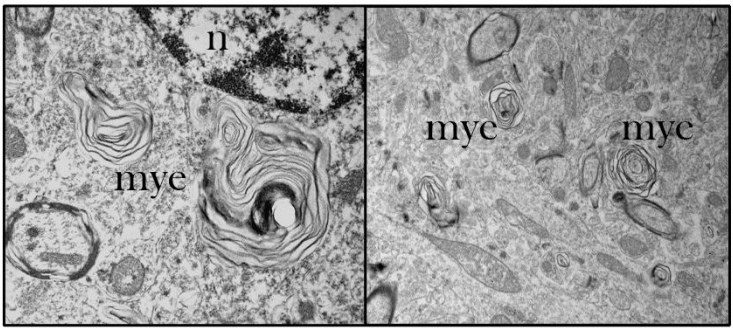

Fig.4.1.3 Ultrastructural detail of a myelin figure (mye) at 12000x from a $TG2^{-/-}$ mouse of 12 months. n, nucleus.

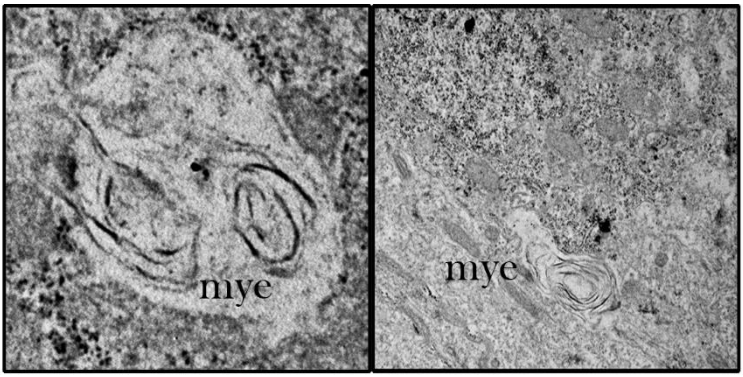

Fig.4.1.4 Ultrastructural detail of a myelin figure (mye) at 12000x from a $TG2^{-/-}$ mouse of 24 months.

(asterisk in Fig.4.1.2, C). This long known marker of damaged cells, while supporting a severe impact of TG2 deficiency on the general cell functioning, suggests specific involvement of the enzyme in the structural maintenance of neuronal nuclear envelope, through interaction with cytoskeletal proteins (Foster et al., 2011). In the CA1 region, representative of the hippocampus, $TG2^{-/-}$ neurons present with similar alterations to the neocortex. In this region,

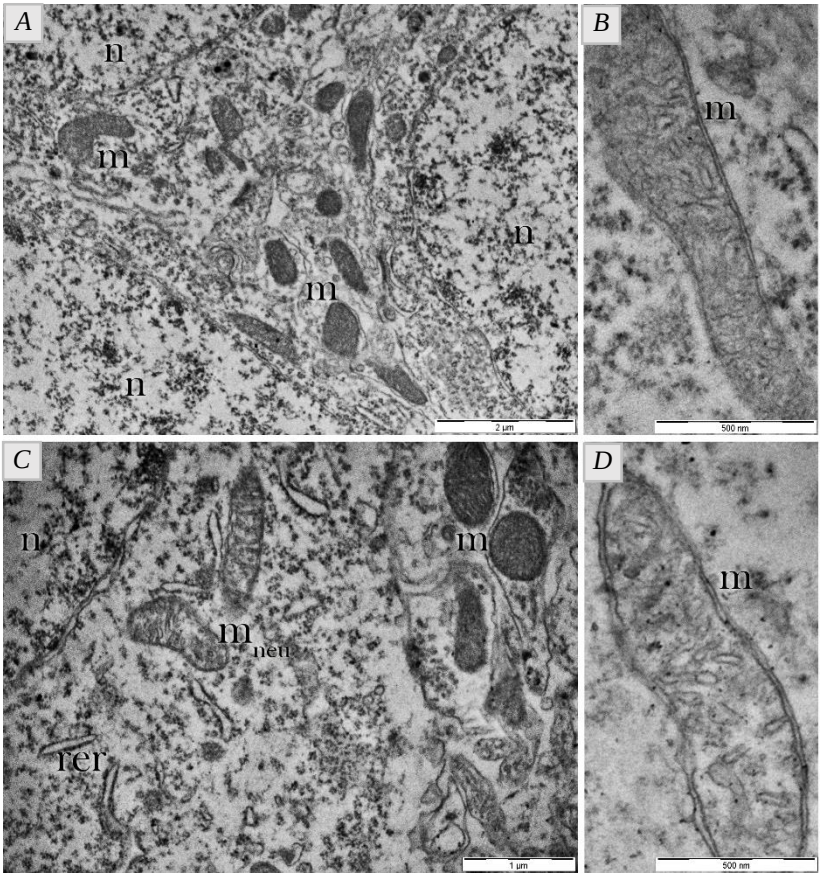

Fig.4.1.5 Ultrastructural analysis of brain CA1 region of the hippocampus from 12 months old mice. (A) Normal morphology of a WT neuronal cell at 11500x magnification (B) Detail of a normal mitochondria (m) from a WT neuronal cell at 31000x magnification (C) Morphology of a $TG2^{-/-}$ neuronal cell, to notice the difference between the injured neuronal mitochondria (m_{neu}) and the normal ones of the intercellular space (m) at 19500x magnification (D) Detail of the damaged mitochondria from a $TG2^{-/-}$ neuronal cell 30000x. Rer, rough endoplasmic reticulum; n, nucleus

where astroglia is especially abundant, a striking feature of knockouts is

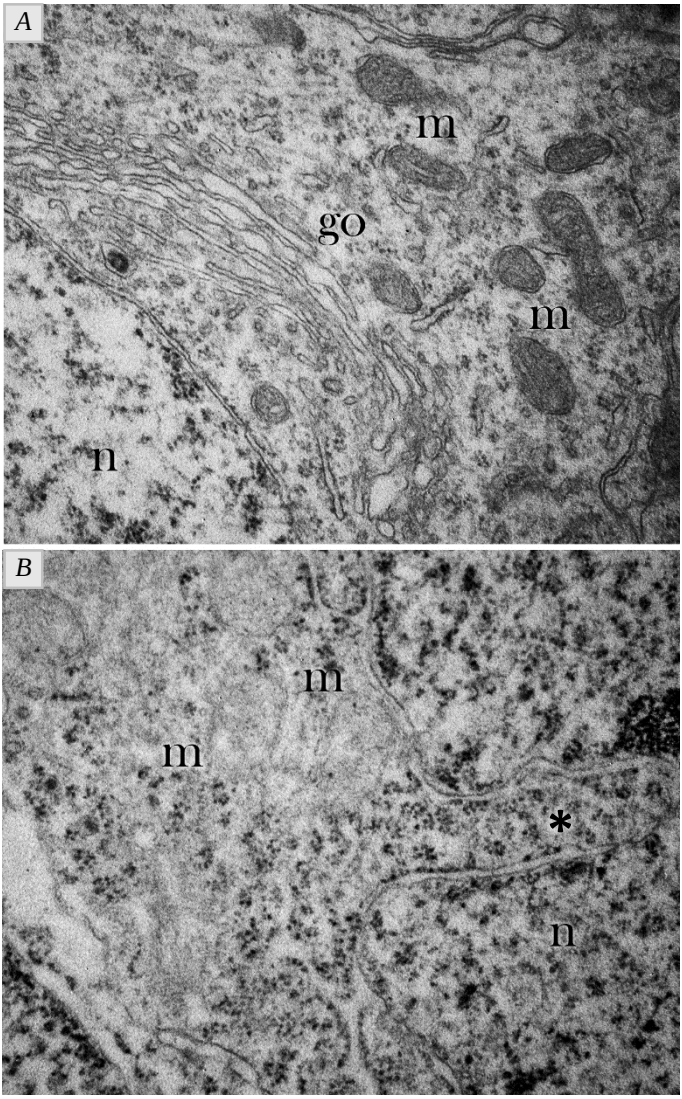

Fig.4.1.6 Ultrastructural analysis of brain CA1 region of the hippocampus from 24 months old mice. (A) Normal morphology of a WT neuronal cell at 30000x magnification (B) Morphology of a TG2$^{-/-}$ neuronal cell, with "ghosts" of mitochondria (m) and nuclear indentation (*) at 30000x. n,nucleus; go, golgi apparatus

apparent, i.e., morphological differences between neuronal and glial cells. Specifically, mitochondria are dramatically damaged in neurons, whilst well preserved in glial cells *(Fig.4.1.5, C)*. Additional neuronal alterations include fragmented endoplasmic reticulum *(Fig.4.1.5, C)*, possibly due to TG2 direct impact on ER function, in particular *via* post-translational modification of ER membrane proteins (Verhaar et al., 2012). At 24 months we detect the same

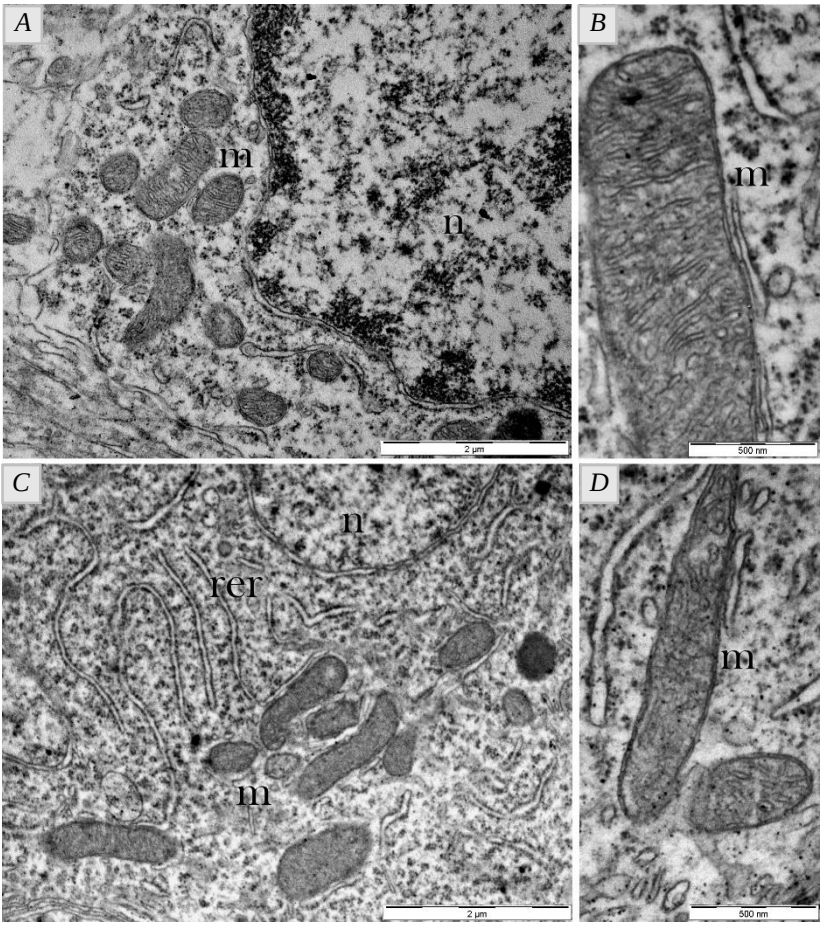

Fig.4.1.7 Ultrastructural analysis of brain cerebellum from 12 months old mice. (A) Normal morphology of a WT purkinje cell at 20000x magnification (B) Detail of a normal mitochondria (m) from a WT purkinje cell at 40000x magnification (C) Morphology of a $TG2^{-/-}$ purkinje cell, at 20000x magnification (D) Detail of the mitochondria from a $TG2^{-/-}$ purkije cell 40000x. Rer, rugose endoplasmic reticulum; n, nucleus

alterations already found in 24-month-old neocortex: ghosts of mitochondria

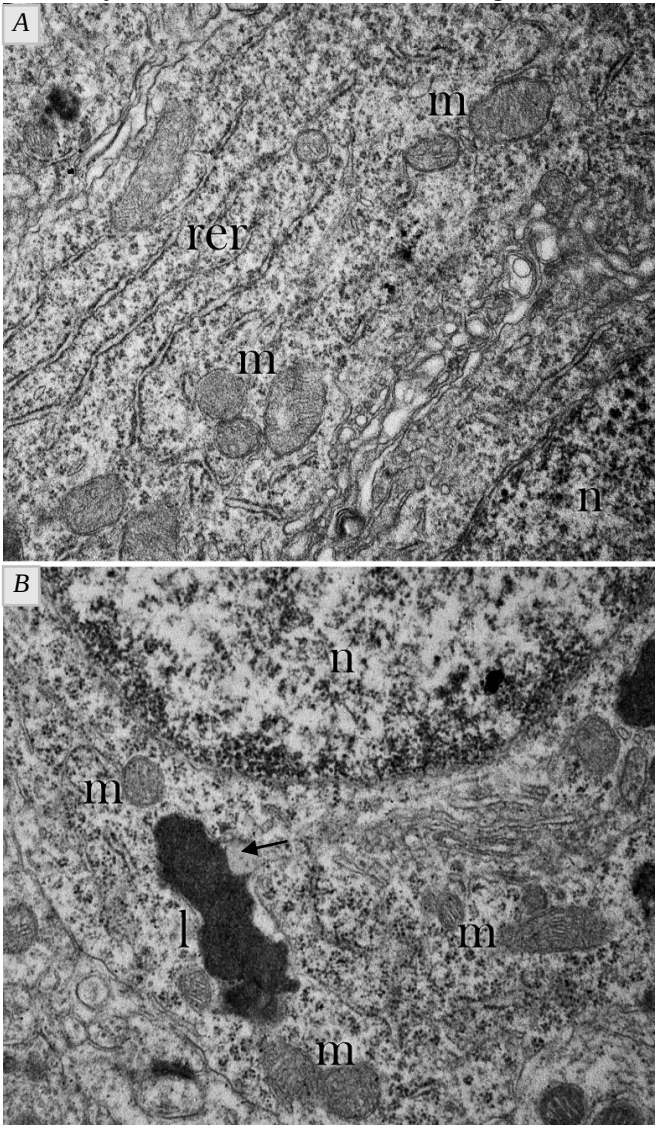

Fig.4.1.8 Ultrastructural analysis of brain cerebellum from 24 months old mice. (A) Normal morphology of a WT purkinje cell at 20000x magnification (B) Morphology of a $TG2^{-/-}$ purkinje cell, at 20000x magnification. Rer, rough endoplasmic reticulum; n, nucleus; l, lipofuscine deposit; m, mitochondria

and nuclear indentation *(Fig.4.1.6, B).*

Last, but not least, the cerebellar cortex shows minor alterations in respect to the other two areas, at both 12 *(Fig.4.1.7)* and 24 months *(Fig.4.1.8).* In fact, mitochondria *(Fig.4.1.7., D)* and endoplasmic reticulum *(Fig.4.1.7, C)* of $TG2^{-/-}$ mice are morphologically more conserved in respect to the ones of the neocortex and hippocampus of the same genotype and age, though slightly altered in respect to the WT *(Fig.4.1.7, A; Fig.4.1.8, A)*, in that we observe lipofuscin formation associated with mitochondria (*Fig.4.1.8, B).*

4.2 Protein immunoanalysis

In order to get a more detailed picture of brain involvement in TG2 deletion, we performed immunoblotting analysis, to quantify representative proteins of autophagy pathway (Beclin1, AMBRA1) and antioxidant response (CAT, GPx1/2, SOD1, SOD2). This investigation was paralleled with immunohistochemical analysis of the same markers, to gain information on their brain distribution and qualitative expression.

TG2 and the autophagy mechanism

Immunoblotting analysis performed in the three selected brain regions overall shows higher levels of expression of Beclin1 and AMBRA1 in the $TG2^{-/-}$ mice in respect to the WT, in accordance to the results obtained for the liver. However, area- and age-dependent patterns of protein expression

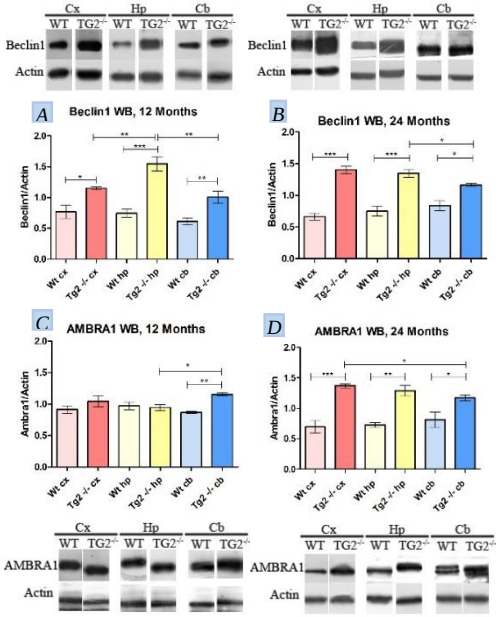

Fig.4.2.1 Densitometric analysis of Beclin1 WB assay at 12 months (A) and 24 months (B) and AMBRA1 WB assay at 12 months (C) and 24 months (D) of brain cortex (cx), hippocampus (hp) and cerebellum (cb) of WT and TG2-/- mice. * p<0,05; **p<0,01; ***p<0,001

can be recognized. Regarding Beclin1, TG2 deletion causes a peculiar increase in its expression: in 12-month-old $TG2^{-/-}$ mice, the hippocampal area shows maximal expression, while in neocortex and cerebellum Beclin1 levels are induced at comparable levels *(Fig.4.2.1, A)*.

At 24 months, TG2$^{-/-}$ neocortical levels of Beclin1 reach those of the hippocampal area, while cerebellar expression remains constant in respect to 12 months *(Fig.4.2.1, B)*. AMBRA1 expression is significantly higher only in the cerebellar area of 12-month-old TG2$^{-/-}$ mice *(Fig.4.2.1, C)*, while at 24 months all the brain regions analyzed show higher expression of the marker in the TG2$^{-/-}$ mice *(Fig.4.2.1, D)*. As seen for Beclin1, the levels of AMBRA1 in the cerebellum of mice aging 24 months is unchanged, compared to 12

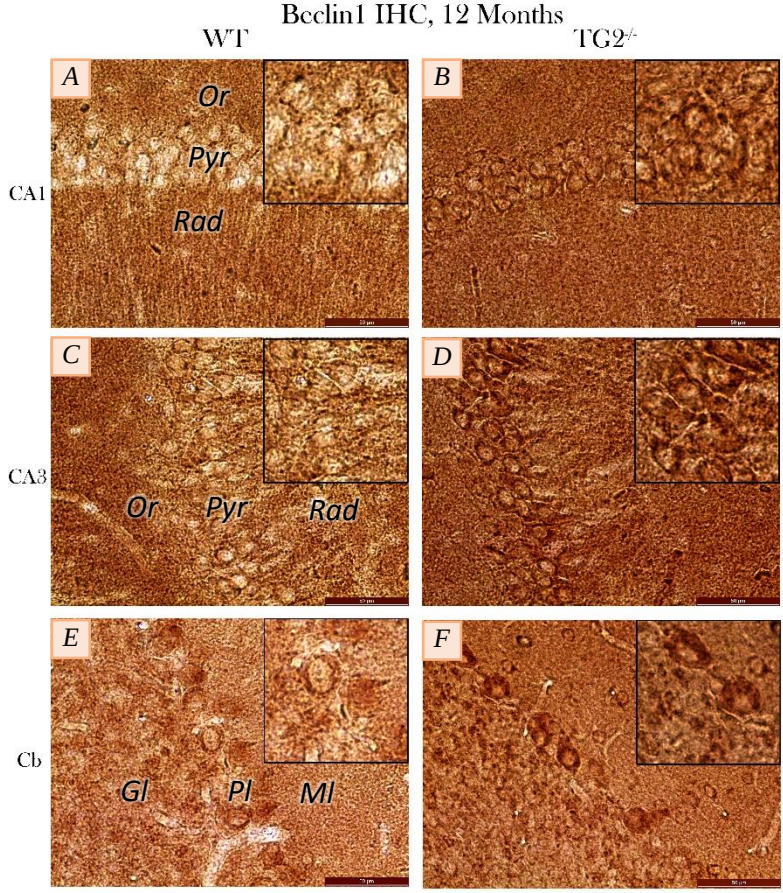

Fig.4.2.2 Beclin1 IHC of 12 months old mice. TG2$^{-/-}$ neocortex (B) and hippocampal (D) pyramidal cells, and Purkinje cell (F) of cerebellar cortex shows a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

months, suggesting this to be a less responsive area. This finding points out the higher rate of autophagy induction in the hippocampal area, probably due to a higher sensitivity of this region to different insults (e.g.oxidative stress, mitochondrial impairment, excitotoxic insults), while the cerebellum seems to be a more resistant region.

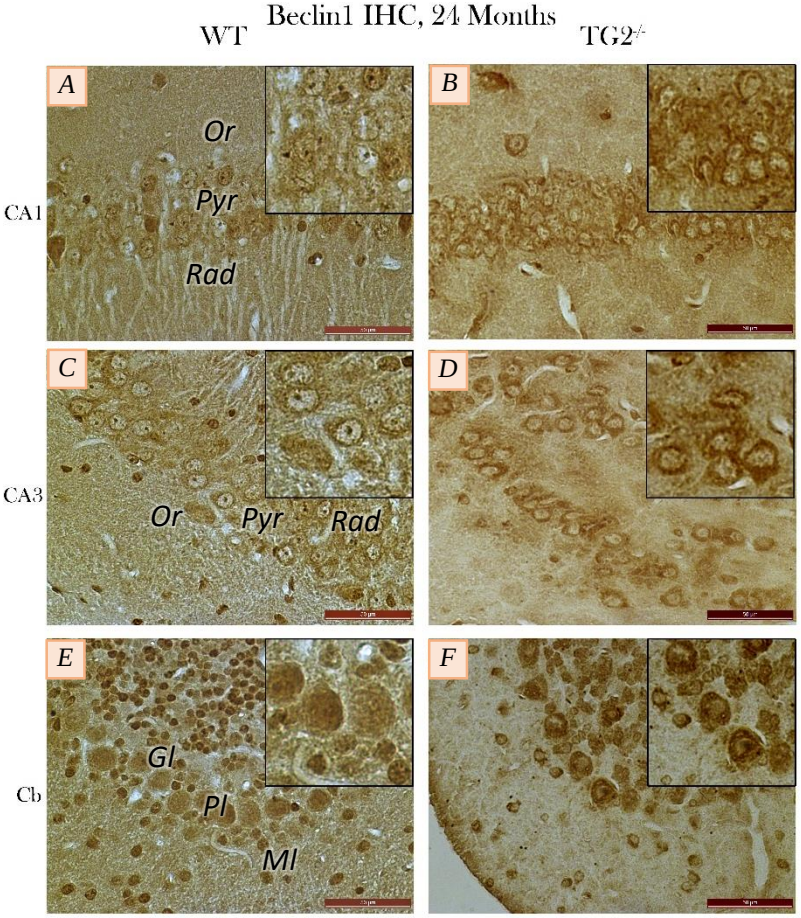

Fig.4.2.3 Beclin1 IHC of 24 months old mice. TG2$^{-/-}$ neocortex (B) and hippocampal (D) pyramidal cells, and Purkinje cell (F) of cerebellar cortex shows a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

The immunohistochemistry analysis is in agreement with immunoblotting results and also with electron microscopic observations. Fig. 4.2.2 shows representative fields of hippocampal formation and cerebellar cortex from 12-month-old mouse brain, immunoreacted for Beclin1. Darker staining is seen in TG2$^{-/-}$ sections as compared to WT, particularly in the pyramidal neurons of hippocampal fields CA1 and CA3 *(Fig.4.2.2, B, D)*, and in

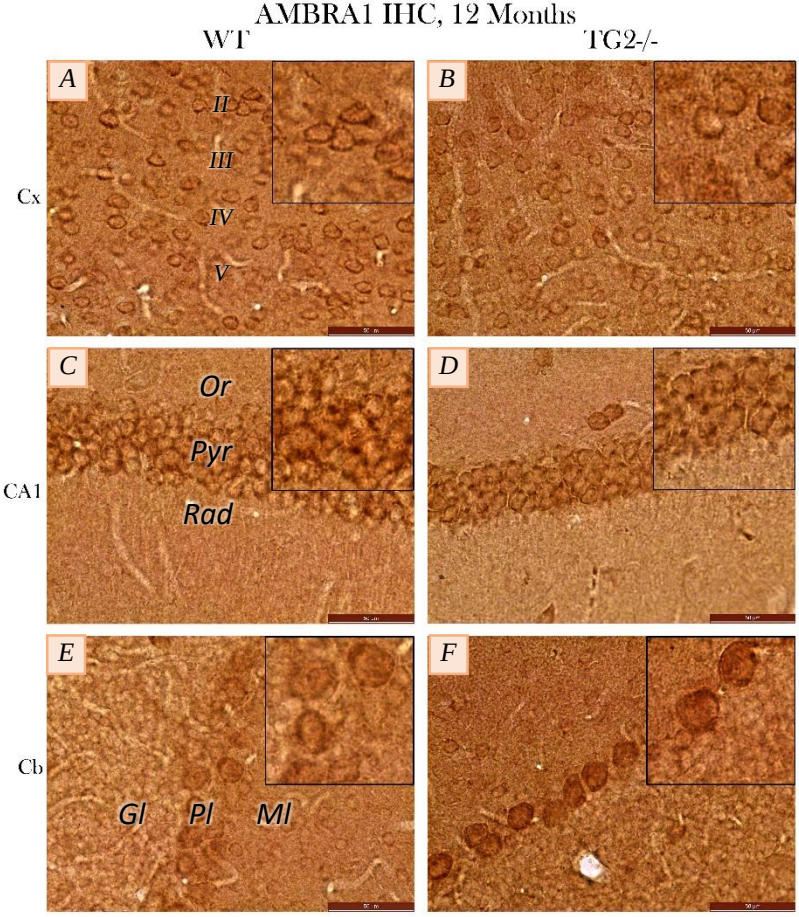

Fig.4.2.4 AMBRA1 IHC of 12 months old mice. TG2$^{-/-}$ Purkinje cell (F) of cerebellar cortex shows a higher staining in respect to WT, while neocortex (B) and hippocampal (D) pyramidal cells, show no significant variation. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

Purkinje cells of the cerebellar cortex *(Fig.4.2.2, F)*. At 24 months, data on overexpression of Beclin1 are confirmed for both areas *(Fig.4.2.3)*, with the additional finding of an evident sufferance of CA1 and CA3 hippocampal regions, which appear thinner than WT and contain morphologically altered neurons, consistent with ultrastructural data (*Fig.4.2.3, B, D*). At this late age, even the cerebellar Purkinje cells show irregular shape and abnormalities in

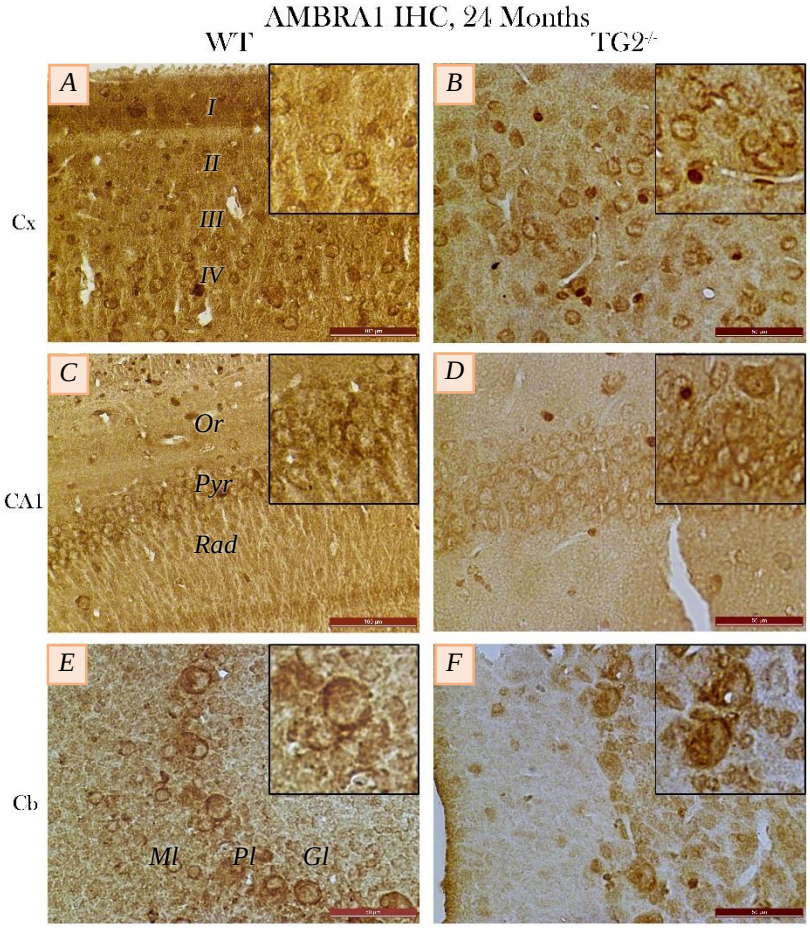

Fig.4.2.5 AMBRA1 IHC of 24 months old mice. $TG2^{-/-}$ neocortex (B) and hippocampal (D) pyramidal cells, and Purkinje cell (F) of cerebellar cortex shows a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

both cytoplasmic and nuclear compartments, consistent with ultrastructural findings (*Fig.4.2.3, F*).

Immmunostaining for AMBRA1 in 12-month-old TG2$^{-/-}$ mice results more intense only in the cerebellar cortex *(Fig.4.2.4, F)*, while at 24 months AMBRA1 *(Fig.4.2.5)* immunostaining is higher in the TG2$^{-/-}$ mice in respect to the WT, irrespective of the brain region considered, and concordantly with the pattern shown by its interactor Beclin1. Indeed, neocortical and hippocampal pyramidal neurons *(Fig.4.2.5, B, D)*, as well as Purkinje cells *(Fig.4.2.5, F)* are highly immunoreactive in TG2-/- mice.

TG2 and the antioxidant response

We started our analysis of the antioxidant response with the superoxide scavenger SOD2, frequently used as a mitochondrial marker, in order to also

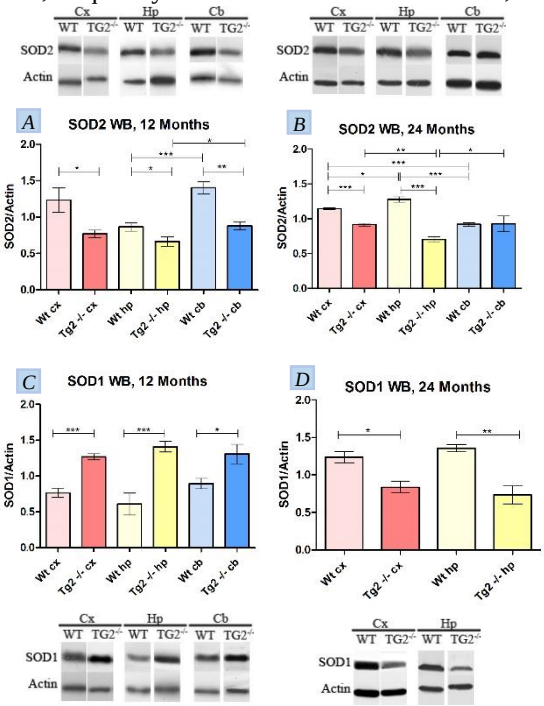

Fig.4.2.6 Densitometric analysis of SOD2 WB assay at 12 months (A) and 24 months (B) and SOD1 assay at 12 months (C) and 24 months (D) of brain cortex (cx), hippocampus (hp) and cerebellum (cb) of WT and TG2-/- mice. * p<0,05; **p<0,01; ***p<0,001

gain information about this cell compartment. As announced by liver and brain ultrastructural analyses, mitochondria are likely to be structurally/functionally injured in TG2 deficient mice. Indeed, SOD2 immunoblotting analysis on both 12- and 24-month-old mice reveal a lower concentration of this marker in $TG2^{-/-}$ brain extracts for all areas considered, with the exception of the cerebellum at 24 months, showing no significant variation in SOD2 expression *(Fig.4.2.6, A, B)*. Immunohistochemistry

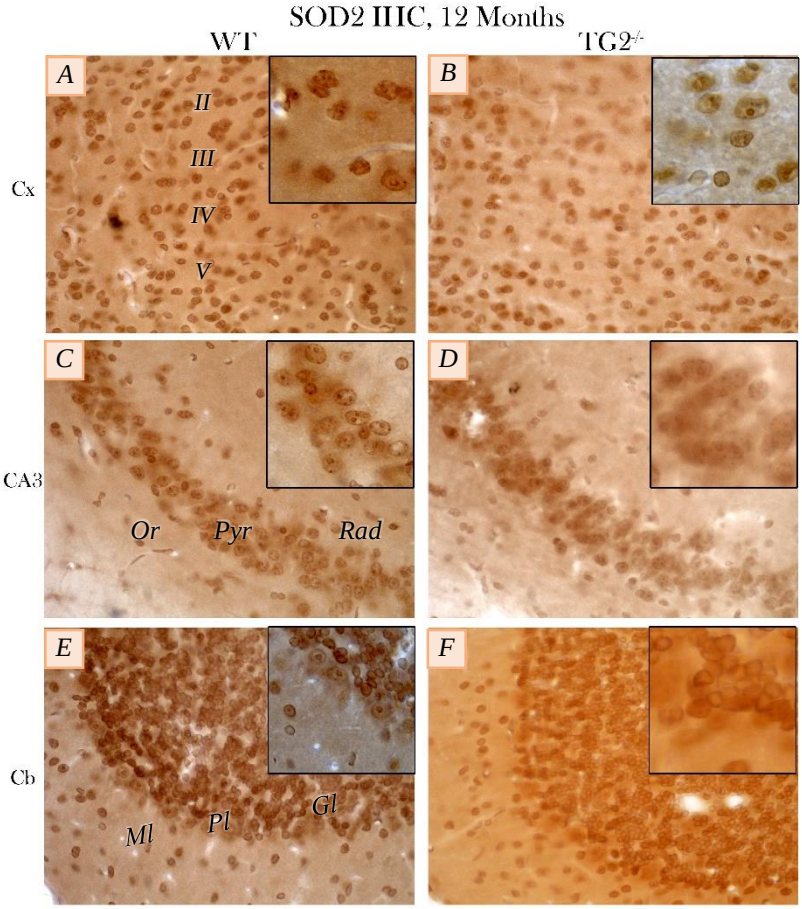

Fig.4.2.7 SOD2 IHC of 12 months old mice. $TG2^{-/-}$ neocortex (B) and hippocampal (D) pyramidal cells, and Purkinje cell (F) of cerebellar cortex shows a lower staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

(Fig.4.2.7, 4.2.8) is in accordance with immunoblotting data, demonstrating overall decreased SOD2 levels, even in cerebellar neurons of 24-month-old TG2$^{-/-}$ animals *(Fig.4.2.8, F)*. The reason for the partial discrepancy with WB data, failing to detect variation in the latter area, may relate to the more precise analysis allowed by IHC. In any case, the putative variations in SOD2 do not seem to affect mitochondrial morphology, as judged by ultrastructural examination of Purkinje cells, which appear as normal *(Fig.4.1.7, 4.1.8)*.

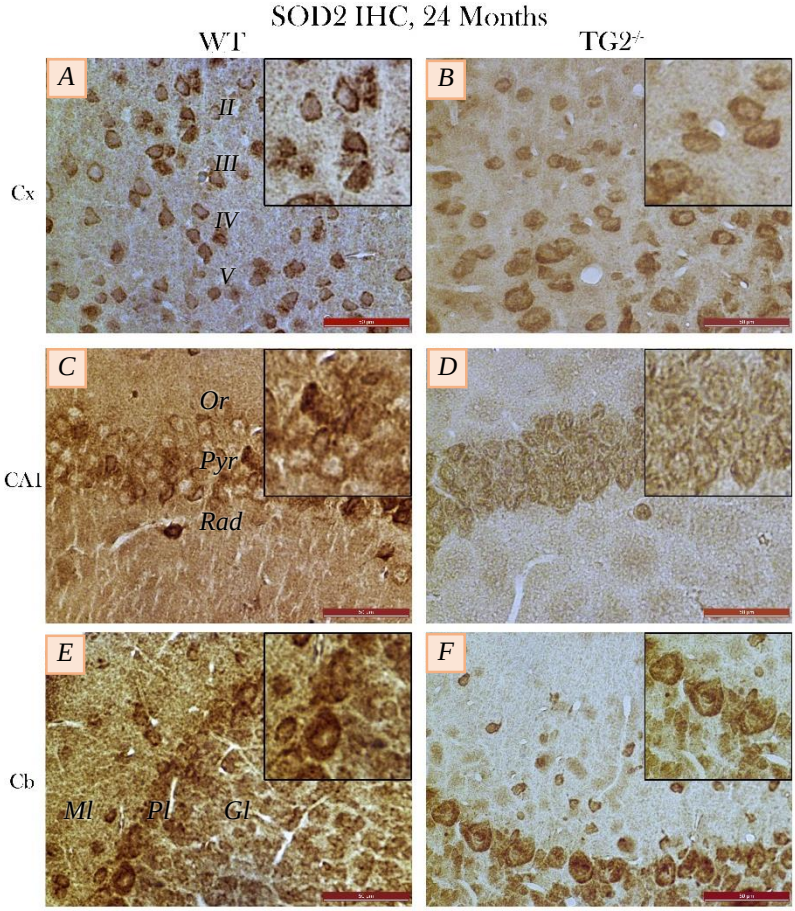

Fig.4.2.8 SOD2 IHC of 24 months old mice. TG2$^{-/-}$ neocortex (B) and hippocampal (D) pyramidal cells, and Purkinje cell (F) of cerebellar cortex shows a lower staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Pl, Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

Immunoblotting analysis shows in 12-month-old TG2$^{-/-}$ brain a consistently higher concentration of SOD1 *(Fig.4.2.6, C)*. This result, similar to that obtained for the liver, argues for an antioxidant response possibly activated by oxidative stress, in turn generated by dysfunctional mitochondria. Conversely, at 24 months we found decreased levels of SOD1, suggestive of severe cell damage and loss of buffering capacities against ROS overload, due to mitochondrial profound damage *(Fig.4.2.6, D)*. It is also possible that progressive damage activates other cellular responses, namely apoptosis. Preliminary data on activated Caspase 3 expression in 12-month-old animals indeed show higher abundance of this major pro-apoptotic protein in TG2$^{-/-}$ mouse neocortex, as compared to WT (*Suppl. Fig. 1*).

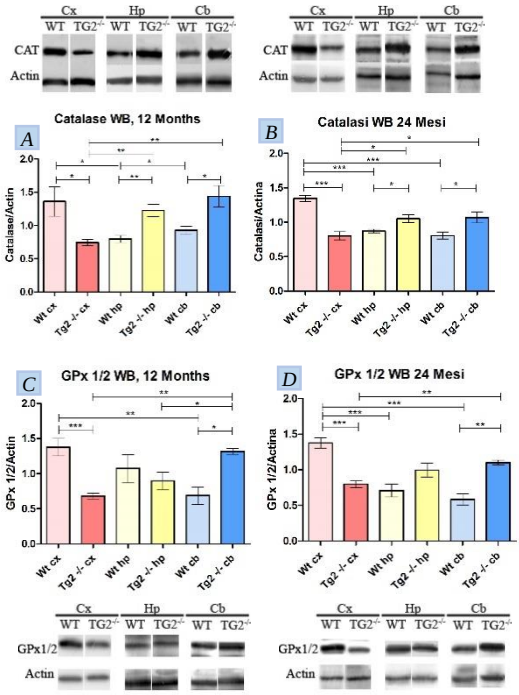

Fig.4.2.9 Densitometric analysis of CAT WB assay at 12 months (A) and 24 months (B) and GPx1/2 assay at 12 months (C) and 24 months (D) of brain cortex (cx), hippocampus (hp) and cerebellum (cb) of WT and TG2-/- mice. * p<0,05; **p<0,01; ***p<0,001

The H_2O_2 detoxifying enzymes CAT and GPx1/2 show a similar pattern of expression in the immunoblotting analysis: they are less expressed in the

neocortex of TG2$^{-/-}$ mice aging 12 or 24 months, while resulting more highly expressed in the cerebellar region. Concerning hippocampal formation, a higher CAT expression in TG2$^{-/-}$ mice is detected, while GPx1/2 content is unchanged *(Fig.4.2.9)*.

The immunohistochemical investigation provides morphological details on the genotype- and age-dependent distribution of CAT and GPx1/2, while qualitatively confirming the immunoblotting data. Lower degree of staining for CAT and GPx1/2 are found in neocortical pyramidal cells *(Fig.4.2.10, B; Fig.4.2.11, B; Fig.4.2.12, B; Fig.4.2.13, B),* while higher immunoreactivity level is observed in cerebellar Purkinje cells *(Fig.4.2.10, F; Fig.4.2.11, F; Fig.4.2.12, F; Fig.4.2.13, F).* An inhomogeneous pattern between the two markers is visible for the hippocampal region, in fact, we find a greater expression of CAT in pyramidal cells *(Fig.4.2.10, D; Fig.4.2.11, D)*, which instead show no variation in GPx1/2 *(Fig.4.2.12, D; Fig.4.2.13, D).* Interestingly, most of the enhanced GPx1/2 immunoreactivity depends on glial cells, especially at 12 months. Thus, TG2 ablation could elicit a response also in neuroglia, consistent with the normal expression of TG2 in both neuronal and glial cells (Wilhelmus et al., 2008). These mechanisms may involve activation of antioxidant defense, allowing us to speculate that at least in some brain areas the inactivation of the protein could lower the neuronal oxidant defense. The observation of a predominant glial staining in the GPx1/2 IHC prompted us to perform immunofluorescence experiments for glial fibrillary acidic protein (GFAP) at both 12 *(Suppl. Fig.2*) and 24 months *(Supll.Fig.3),* in order to gain some information on the glial status. As expected, we can see a higher immunoreactivity in the hippocampus of the TG2$^{-/-}$, mainly at 12 months *(Fig.2, B, D*), even though present also at 24 months (*Fig.3, B, D).*

The above results may be interpreted in view of differential resistance/reactivity/susceptibility to oxidative stress of the examined brain regions. Particularly, the increase in SOD1 observed in the neocortex at 12 months, likely leading to overproduction of H_2O_2, not accompanied by an equal enhancement of hydrogen peroxide scavenging systems, indicates exacerbating redox imbalance. Conversely, the cerebellar region of TG2$^{-/-}$ mice features elevated levels of SOD1, CAT and GPx 1/2, suggesting relatively good management of oxidative stress. As to the hippocampal formation, it can be conceived that at 12 months the area is capable of coping with both $O2^{-\cdot}$ and H_2O_2 overload, while at 24 months defenses against ROS

become insufficient. This hypothesis overall goes along with ultrastructural data, pointing to minor damage caused by TG2 ablation to the cerebellar cortex, and major involvement of neocortical and hippocampal areas.

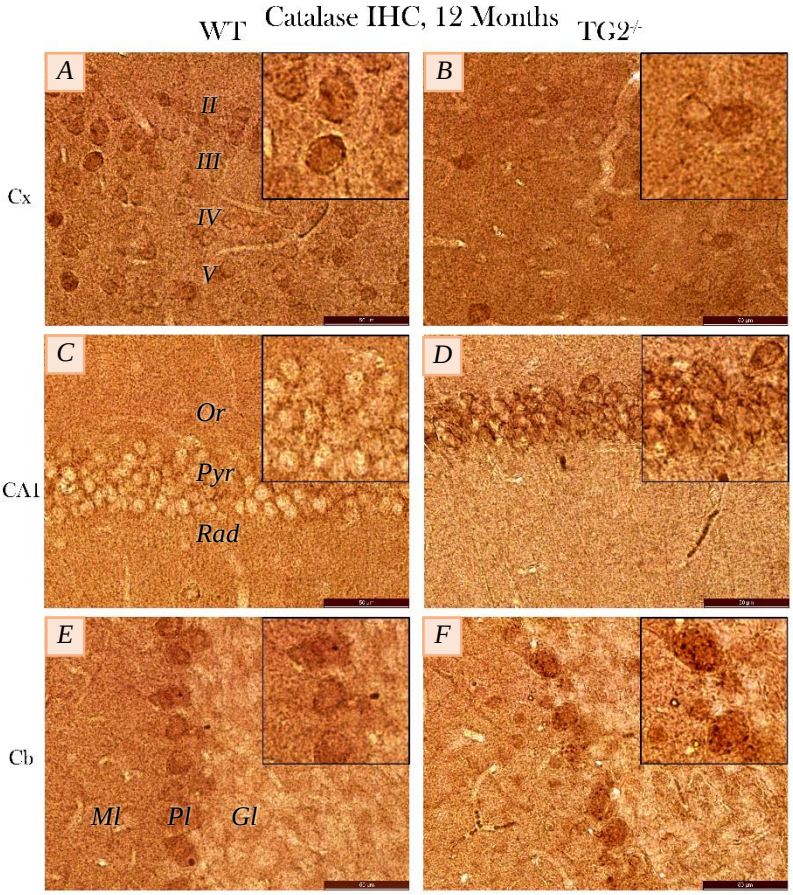

Fig.4.2.10 CAT IHC of 12 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while pyramidal hippocampal (D) and Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

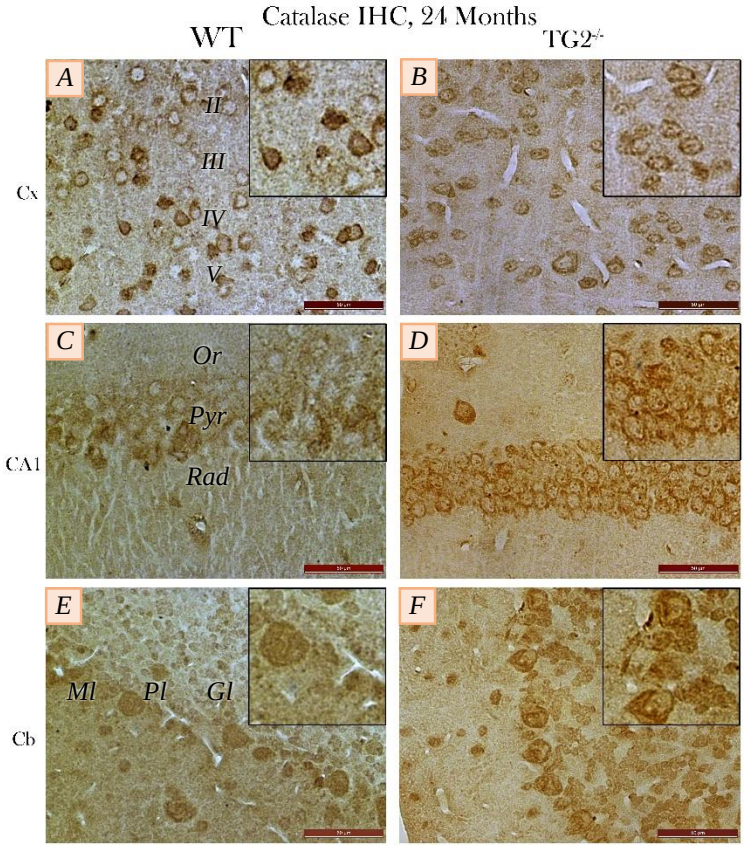

Fig.4.2.11 CAT IHC of 24 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while pyramidal hippocampal (D) and Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

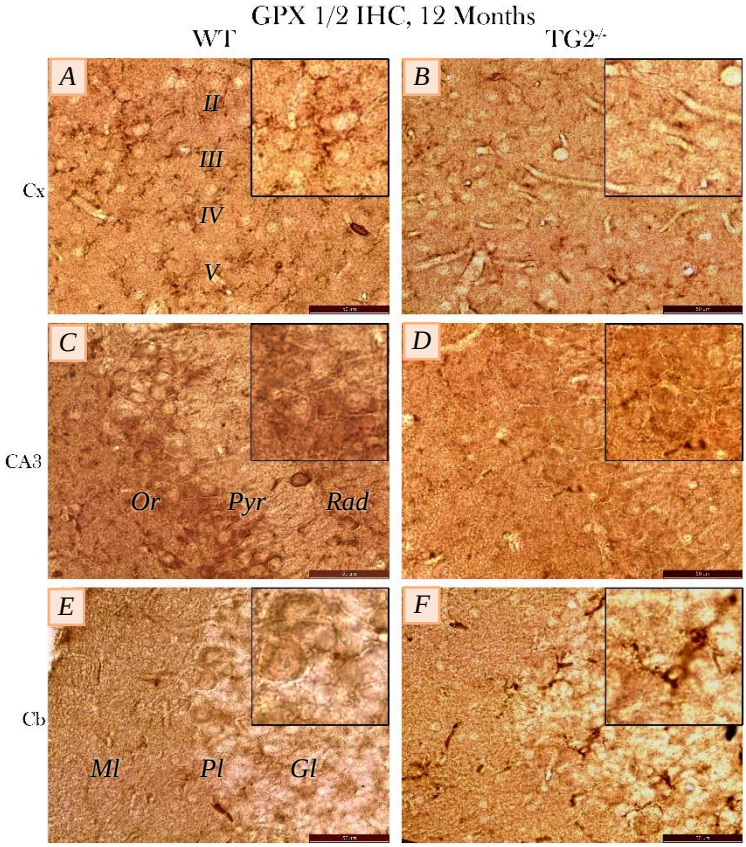

Fig.4.2.12 GPx1/2 IHC of 12 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. No significant variations is visible in the $TG2^{-/-}$ CA3 hippocampal region. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

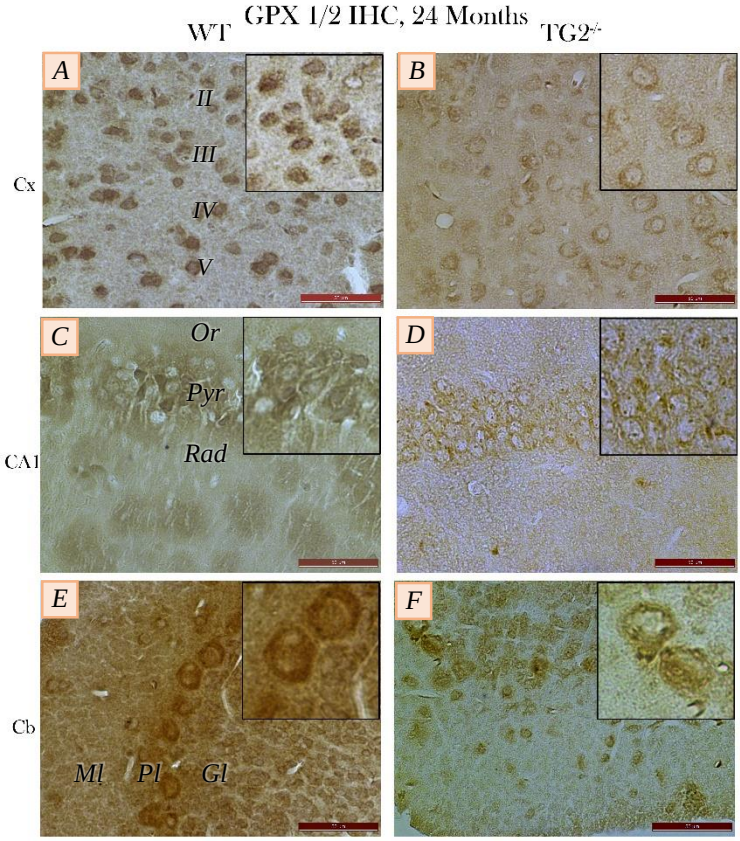

Fig.4.2.13 GPx1/2 IHC of 24 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. No significant variations is visible in the $TG2^{-/-}$ CA3 hippocampal region. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

Our study, though not focused on the analysis of peroxisomes, was extended to a marker of these organelles, in order to understand whether the modulation of CAT expression was paralleled by a modulation of the overall peroxisomal population. We thus performed Western blotting and IHC analyses for 70-kDa peroxisomal membrane protein (PMP70), major component of peroxisomal membranes. Immunoblotting data generally overlap CAT data, at both 12 and 24 months *(Fig.4.2.14)*. Indeed, the neocortex shows a decrease in the marker expression in the TG2$^{-/-}$ mice, while hippocampus and cerebellum show a higher expression in respect to the WT.

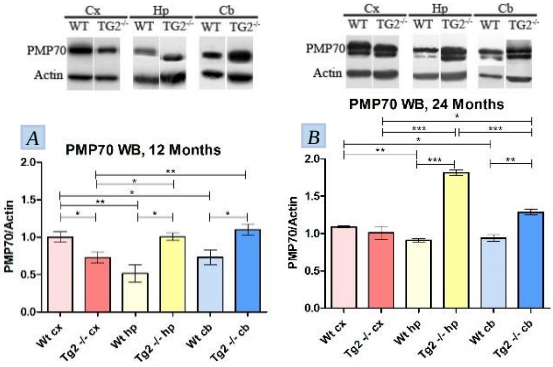

Fig. 4.2.14 Densitometric analysis of PMP70 WB assay at 12 months (A) and 24 months (B) of brain cortex (cx), hippocampus (hp) and cerebellum (cb) of WT and TG2-/- mice. * p<0,05; **p<0,01; ***p<0,001

The immunohistochemistry goes along with the immunoblotting data, it is indeed even more precise, in revealing the same trend for both ages. At 12 and 24 months in fact a lower staining in the TG2$^{-/-}$pyramidal neurons of neocortex area is detected *(Fig.4.2.15, B; 4.2.16, B)*, while hippocampal pyramidal neurons *(Fig.4.2.15, D; 4.2.16, D)* and cerebellar Purkinje cell *(Fig.4.2.15, F; 4.2.16, F)* are more intensely stained in respect to the WT. This results points out that the increase of CAT protein levels in TG2$^{-/-}$ could be probably due to a rise of peroxisomal population, possibly induced by a higher oxidative stress following TG2 ablation. Such mechanism is also suggested by the tight functional relationship linking peroxisomes with mitochondria. This connection involves several aspects of metabolism, ranging from ROS production/scavenging to lipid catabolism/anabolism. Importantly, when one of these cell compartments is dysregulated, the biogenesis and function of the other is typically induced (Demarquoy & Le

Borgne, 2015). Therefore, the occurrence of mitochondrial disturbances may well influence the proliferation of peroxisomal population in specific brain areas. Future studies aimed at further characterizing mitochondrial and peroxisomal functions/dysfunctions in $TG2^{-/-}$ genotype, possibly through

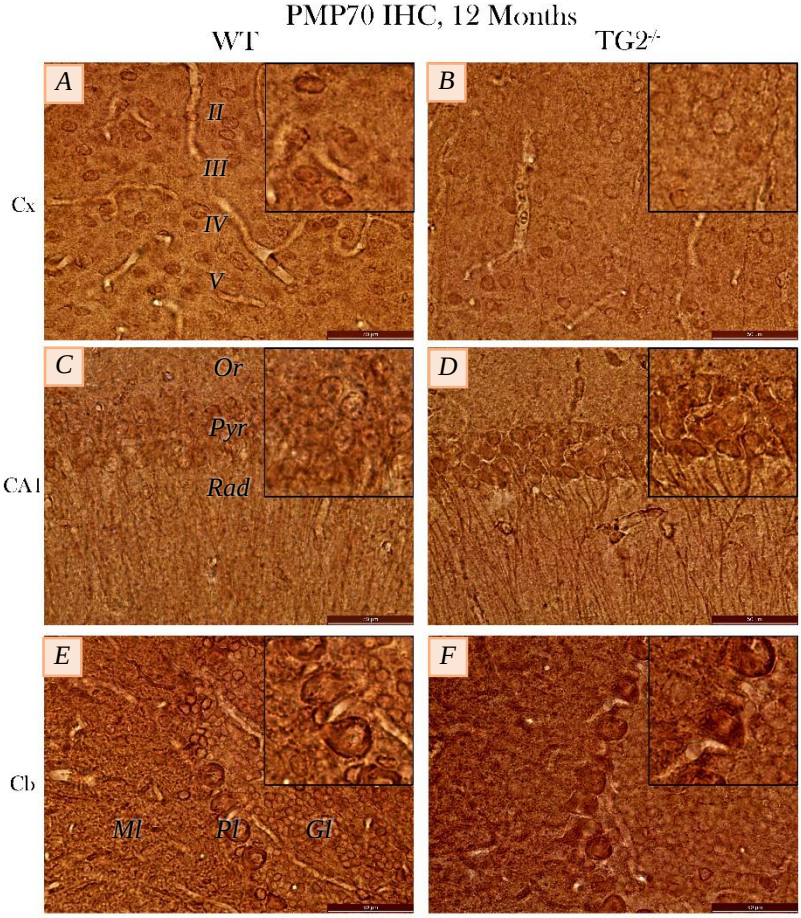

Fig. 4.2.15 PMP70 IHC of 12 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while pyramidal hippocampal (D) and Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

investigation of the transcription factors/cofactors regulating the biogenesis of these organelles are needed to confirm this hypothesis.

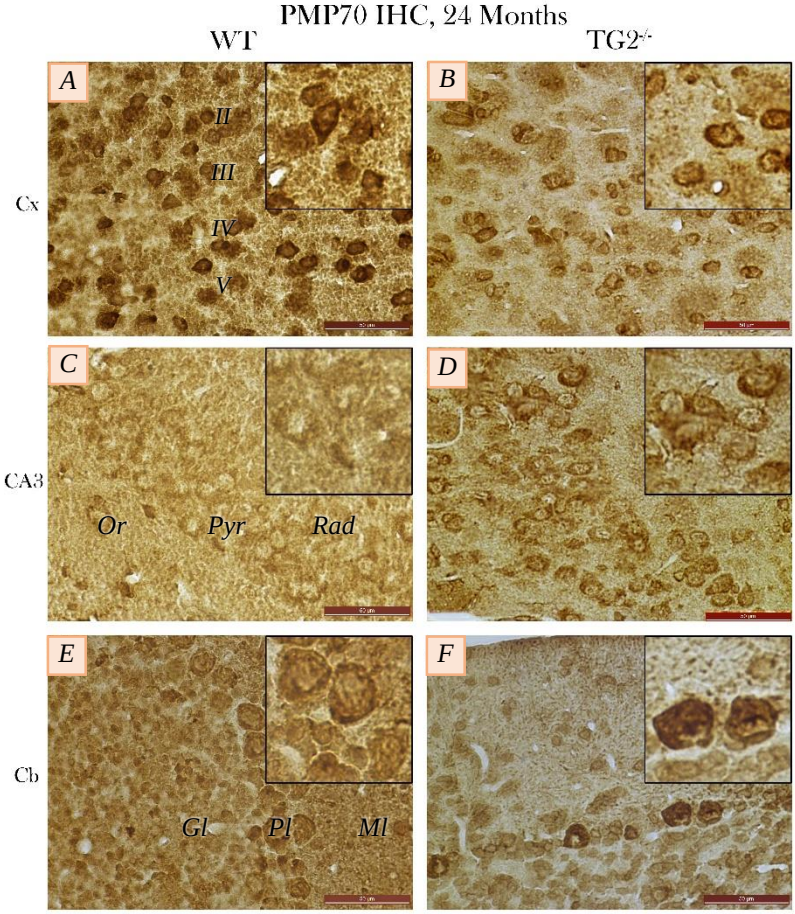

Fig. 4.2.16 PMP70 IHC of 24 months old mice. $TG2^{-/-}$ neocortex pyramidal cells (B), show a lower immunostaining, while pyramidal hippocampal (D) and Purkinje cerebellar cortex cell (F) show a higher staining in respect to WT. Or, stratum oriens; Pyr, pyramidal layer; Rad, stratum radiatum; Purkinje layer; Gl, Granule layer; Ml, Molecular layer; neocortex (cx); hippocampal region (CA3); cerebellar cortex (Cb)

Section III:
Conclusions and perspectives

Chapter 5

Conclusions

The present study is the first to report a thorough investigation on the consequences of TG2 deletion in the brain, in a physiological context. Indeed, previous reports focused on specific aspects of TG2 role in select neuropathologies, markedly neurodegenerative diseases, by examining TG2 contribution in specific pathogenetic mechanisms. By examining diverse brain areas and neuronal populations, from a molecular and morphological point of view, our comprehensive work sheds light into the general role of TG2 in the nervous tissue, while highlighting peculiar region- and cell-specific functions of the protein.

TG2 is the principal isoform in of the TGs family expressed in the brain, present at high levels particularly in the neocortex, hippocampus and cerebellum (Appelt et al., 1996; Johnson et al., 1997; Kim et al., 1999; Lesort et al., 1999, 2000; Andringa et al., 2004; Wilhelmus et al., 2008). TG2 has been implicated in a plethora of different cellular processes, including those reported to be highly altered in the ageing processes, such as apoptosis, autophagy, redox maintenance and inflammation. The direct link between TG2 and the ageing process is strengthened by additional findings: (i) an increase of TG2 mRNA levels have been observed during normal aging; (ii) TG2 has been involved in age-related diseases, including cardiovascular, cancer, hepatic and neurodegenerative pathologies.

Our work started from this premises, with the broad aim to understand TG2 contribution in those processes of brain homeostasis connected with the cellular ageing. In particular, we chose to examine the expression of molecules related to the autophagic pathway and to the response to the oxidative stress, basing on the literature, stressing TG2 contribution to the modulation of these cellular processes *in vitro*. Our results obtained *in vivo*, by a combined molecular/morphological approach converge in demonstrating significant alterations to both pathways in adult and senescent $TG2^{-/-}$ mouse liver and brain. These modifications were contextualized in a fine morphological analysis, conducted by transmission electron microscopic techniques, which highlighted additional altered features in subcellular compartments of TG2 deficient organs.

Both WB and IHC results highlighted conspicuous *in vivo* autophagic induction in the liver and brain from $TG2^{-/-}$ mice. This response, consisting in overexpression of two major regulatory molecules, namely Beclin1 and AMBRA1, is suggestive of an attempt to remove damaged organelles, particularly mitochondria, which show the most prominent ultrastructural alterations. Thus, our data provide *in vivo* evidence of a negative modulatory role of TG2 in the induction phase of autophagy, so far only supported by *in vitro* literature (Akar et al., 2007; Ozpolat et al., 2007; Luciani et al., 2010).

Noteworthy, induction of autophagy in TG2-/- mouse brain appears not to be followed by successful completion of the process. This statement is mainly supported by electron microscopic findings concerning (i) accumulation in $TG2^{-/-}$ mouse brain of mitochondria/lipofuscin aggregates, index of decreased lysosomal degradation, (ii) presence of intracellular "myelin figures", reminiscent of residual autophagic bodies, and (iii) deranged Golgi apparatus, sign of lysosomal disturbed biogenesis. To this respect, it should be taken into account that TG2 is reportedly needed also for the autophagosome-lysosome fusion (D'Eletto et al., 2009). Therefore, even for this aspect of autophagy regulation, our data are the first to demonstrate *in vivo* a function of TG2 in the final stages of the process. Future studies are needed to deepen and dissect the molecular mechanisms of TG2 action in the different steps of the pathway.

Besides providing evidence of disturbed autophagy, ultrastructural data highlighted profound alterations of the mitochondrial compartment subsequent to TG2 ablation, consistent with the role of the enzyme in stabilizing complex I and II of the electron respiratory chain (Malorni et al., 2009). The possible impairment of this fundamental mitochondrial component likely leads to superoxide anion leakage. The effects of accumulation of this toxic molecule appear aggravated by the decreased expression of its major scavenger SOD2. This likely leads to a vicious circle of energetic imbalance and oxidative stress, perpetuating mitochondrial damage and propagating the insult to the whole cell and tissue. It should be nevertheless noted that the above described condition applies to the neocortex and, partly, to the hippocampal formation, regions where ROS detoxifying enzymes are downregulated, suggesting these areas to be prone to oxidative stress. By contrast, it is interesting to note how cerebellar area, long known to be a more protected region of the brain in many neurodegenerative diseases, displays less damaged mitochondria in $TG2^{-/-}$ mice. This could

relate to an efficient activation of the antioxidant systems – indeed we detected higher expression of H_2O_2 metabolizing enzymes CAT and GPx 1/2- and/or to a higher autophagic flux. Interestingly, response to cellular stress caused by TG2 ablation apparently involves peroxisomes, which are biogenetically induced -as judged by increased PMP70 immunoreactivity – possibly to cope with mitochondrial deficiency, as it occurs in other pathophysiological situations. Notably, this induction strongly occurs in the cerebellum, indicating a protective role for these often neglected organelles.

Thus, our study confirms *in vivo* an important role of TG2 in regulating energy metabolism and in maintaining redox homeostasis in the brain and supports a correlation between TG2 and the antioxidant response, which is however strongly dependent on the area considered (Fesus & Szondy 2005).

Nevertheless, our data clearly show that any attempt by the cell to counteract cell injury consequent to TG2 ablation, is subject to progressive loss of efficiency when senescence ensues. When comparatively analyzing results from 12- and 24-month-old mice, a worsening of antioxidant defences and autophagic efficiency, associated with ultrastructural damage is detected, even in regions (cerebellum) more resistant to insult.

Chapter 6

Future perspectives

Our study, by providing novel insights into previously suggested TG2 roles in the brain, and especially by highlighting new functions of the protein in the complex organ, opens the way to future mechanistic investigations, which will hopefully lead to therapeutic applications.

One critical aspect requiring further deepening concerns the precise TG2 role in the autophagic process. It should be possible to generate GFP-RFP-LC3-TG2$^{-/-}$ transgenic mice that could be used to monitor autophagic flux *in vivo*. This tandem construct morphologically traces autophagic flux, by labelling both autophagosomes and autolysosomes with different fluorocromes, thus permitting to recognise if the maturation step is blocked or increased (Kimura et al., 2007). This type of experiments could either be conducted in a physiological context, with ageing as unique variable, or done in a rapamycin -an inducer of autophagy- administration setting, or even in a neuropathological background.

Particularly interesting will be crossing these genetically modified mice with models of neurodegenerative disorders, which are presently being studied at the laboratory where I have carried out my PhD project. Among these, Alzheimer's disease Tg2576 strain, displaying autophagic disturbances and oxidative stress early in the pathology, is a promising subject of study (Fanelli et al., 2013; Sepe et al., 2014; Porcellotti et al., 2015).

To deepen the analysis of the relationship linking TG2 with redox maintenance, oxidative stress damage should be characterized by looking for parameters of lipid peroxidation, oxidative protein and nucleic acids modification in TG2-/- mouse brain, Moreover, redox sensing transcription factors (e.g., NRF2, PGC1alpha), regulating antioxidant response should also be investigated.

While possible mitochondrial DNA damage should be tested by 8-hydroxy guanosine detection, in order to confirm this mitochondrial injury/alteration, it could also be useful to perform *ex vivo* biochemical study on the mitochondrial activity: mitochondrial fuel usage, oxygen consumption rate, assessing of the key parameters of glycolytic flux.

Further studies are also needed to clarify the contribution of glial cells in the $TG2^{-/-}$ phenotype, to highlight possible specific roles of the protein in this cell type, an issue so far unexplored.

Once acquired the above knowledge, therapeutic approaches involving modulation of TG2 expression and activity during aging and age-related pathologies can hopefully be envisioned to slow down or prevent neurodegeneration.

References

1) Achyuthan KE & Greenberg CS (1987) **Identification of a guanosine triphosphatebinding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity**. *J Biol Chem* 262(4):1901–1906
2) Akar U, Ozpolat B, Mehta K, Fok J, Kondo Y, Lopez-Berestein G (2007) **Tissue transglutaminase inhibits autophagy in pancreatic cancer cells**. *Mol Cancer Res* 5(3):241–249

3) Altuntas S, D'Eletto M, Rossin F, Hidalgo LD, Farrace MG, Falasca L, Piredda L, Cocco S, Mastroberardino PG, Piacentini M, Campanella M (2014) **Type 2 Transglutaminase, mitochondria and Huntington's disease: menage a trois**. *Mitochondrion*. Pt A:97-104

4) Andringa G, Lam KY, Chegary M, Wang X, Chase TN, Bennett MC (2004) **Tissue transglutaminase catalyzes the formation of alpha-synuclein crosslinks in Parkinson's disease**. *FASEB J.* 18:932–934

5) Antonyak MA, Singh US, Lee DA, Boehm JE, Combs C, Zgola MM, Page RL, Cerione RA (2001) **Effects of tissue transglutaminase on retinoic acid-induced cellular differentiation and protection against apoptosis**. *J. Biol. Chem.* 276:33582–33587.

6) Bailey CD & Johnson GV (2005) **Tissue transglutaminase contributes to disease progression in the R6/2 Huntington's disease mouse model via aggregate-independent mechanisms**. *J. Neurochem*. 92:83–92

7) Battaglia G, Farrace MG, Mastroberardino PG, Viti I, Fimia GM, Van Beeumen J, Devreese B, Melino G, Molinaro G, Busceti CL, Biagioni F, Nicoletti F, Piacentini M (2007**) Transglutaminase 2 ablation leads to defective function of mitochondrial respiratory complex I affecting neuronal vulnerability in experimental models of extrapyramidal disorders.** *J Neurochem* 100(1):36–49

8) Belkin AM (2011) **Extracellular TG2: emerging functions and regulation**. *FEBS J*. 278(24):4704-16

9) Bernassola F, Federici M, Corazzari M, Terrinoni A, Hribal ML, De Laurenzi V, Ranalli M, Massa O, Sesti G, McLean WH, Citro G, Barbetti F, Melino G (2002) **Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient**. *FASEB* **J** 16(11):1371–1378

10) Budillon A, Carbone C, Di Gennaro E (2013) **Tissue transglutaminase: a new target to reverse cancer drug resistance.** *Amino Acids*. 44(1):63-72

11) Caccamo D, Campisi A, Curro M, Li Volti G, Vanella A, Ientile R (2004) **Excitotoxic and post-ischemic neurodegeneration: involvement of transglutaminases**. **Amino Acids** 27:373–379

12) Caccamo D, Campisi A, Curro M, Bramanti V, Tringali M, Li Volti G, Vanella A, Ientile R (2005a) **Antioxidant treatment inhibited glutamate-evoked NF-kappaB activation in primary astroglial cell cultures.** *Neurotoxicology* 26:915–921

13) Caccamo D, Campisi A, Marini H, Adamo EB, Li Volti G, Squadrito F, Ientile R (2005b) **Glutamate promotes NF-kappaB pathway in primary astrocytes: protective effects of IRFI 016, a synthetic vitamin E analogue**. *Exp Neurol* 193:377–383

14) Caccamo D, Campisi A, Curro M, Aguennouz M, Li Volti G, Avola R, Ientile R (2005c) **Nuclear factor-kappab activation is associated with glutamate-evoked tissue transglutaminase upregulation in primary astrocyte cultures**. *J Neurosci Res* 82:858–865

15) Caccamo D, Curro M, Condello S, Ferlazzo N, Ientile R, Caccamo D (2010) **Critical role of transglutaminase and other stress proteins during neurodegenerative processes.** *Amino Acids* 38(2):653– 658

16) Caccamo D, Currò M, Ferlazzo N, Condello S, Ientile R (2012) **Monitoring of transglutaminase 2 under different oxidative stress conditions**. *Amino Acids* 4:1037–1043

17) Campisi A, Caccamo D, Li Volti G, Curro M, Parisi G, Avola R, Vanella A, Ientile R (2004) **Glutamate-evoked redox state alterations are involved in tissue transglutaminase up-regulation in primary astrocyte cultures**. *FEBS Lett* 578:80–84

18) Chakraborty G, Leach T, Zanakis MF, Sturman JA, Ingoglia NA (**1987) Posttranslational protein modification by polyamines in intact and regenerating nerves**. *J. Neurochem*. 48:669-675

19) Chen, JS & Mehta K (1999) **Tissue transglutaminase: an enzyme with a split personality**. *Int J Biochem Cell Biol* 31(8):817–836

20) Chiocca EA, Davies PJ, Stein JP (1989) **Regulation of tissue transglutaminase gene expression as a molecular model for retinoid effects on proliferation and differentiation**. *J. Cell. Biochem*. 39:293–304

21) Cimini A, Moreno S, D'Amelio M, Cristiano L, D'Angelo B, Falone S, Benedetti E, Carrara P, Fanelli F, Cecconi F, Amicarelli F, Cerù MP (2009) **Early biochemical and morphological modifications in the brain of a transgenic mouse model of Alzheimer's disease: a role for peroxisomes**. *J Alzheimers Dis*.18(4):935-52

22) D'Eletto M, Farrace MG, Falasca L, Reali V, Oliverio S, Melino G, Griffin M, Fimia GM, Piacentini M (2009) **Transglutaminase 2 is involved in autophagosome maturation**. *Autophagy* 5(8):1145–1154

23) De Laurenzi V & Melino G (2001) **Gene disruption of tissue transglutaminase.** *Mol Cell Biol* 21(1):148–155

24) Demarquoy J & Le Borgne F (2015) **Crosstalk between mitochondria and peroxisomes**. *World J Biol Chem*. 26;6(4):301-309

25) Esposito C, Marra M, Giuberti G, D'Alessandro AM, Porta R, Cozzolino A, Caraglia M, Abbruzzese A (2003) **Ubiquitination of tissue transglutaminase is modulated by interferon alpha in human lung cancer cells.** *Biochem. J.* 370:205–212

26) Fanelli F, Sepe S, D'Amelio M, Bernardi C, Cristiano L, Cimini A, Cecconi F, Ceru' MP, Moreno S (2013) A**ge-dependent roles of peroxisomes in the hippocampus of a transgenic mouse model of Alzheimer's disease**.. *Mol Neurodegener*. 2;8:8

27) Festoff BW, SantaCruz K, Arnold PM, Sebastian CT, Davies PJ, Citron BA (2002) **Injury-induced 'switch' from GTP-regulated to novel GTP-independent isoform of tissue transglutaminase in the rat spinal cord.** *J. Neurochem*. 81: 708–718

28) Fesus L (1992) **Apoptosis**. *Immunol*. *Today*. 13:A16–A17

29) Fesus L & Piacentini M (2002) **Transglutaminase 2: an enigmatic enzyme with diverse functions**. *Trends Biochem Sci* 27(10):534–539

30) Fesus L & Szondy Z (2005) **Transglutaminase 2 in the balance of cell death and survival**. *FEBS Lett*. 579:3297–3302

31) Foster CR, Robson JL, Simon WJ, Twigg J, Cruikshank D, Wilson RG, Hutchison CJ (2011) **The role of Lamin A in cytoskeleton organization in colorectal cancer cells: a proteomic investigation**. *Nucleus*. 2(5):434-443

32) Friedrich P, Fesus L, Tarcsa E, Czeh G (1991) **Protein crosslinking by transglutaminase induced in long-term potentiation in the Ca1 region of hippocampal slices**. *Neuroscience* 43:331–334

33) Giandomenico V, Lancillotti F, Fiorucci G, Percario ZA, Rivabene R, Malorni W, Affabris E, Romeo G (1997) **Retinoic acid and IFN inhibition of cell proliferation is associated with apoptosis in squamous carcinoma cell lines: role of IRF-1 and TGase II-dependent pathways.** *Cell Growth Differ.* 8:91–100

34) Gilad GM & Varon LE (1985a) **Transglutaminase activity in rat brain: characterization, distribution, and changes with age**. *J. Neurochem*. 45:1522-1526

35) Gilad GM, Varon LE, Gilad VH (1985b) **Calcium-dependent transglutaminase of rat sympathetic ganglion in development and afternerve injury**. *J. Neurochem*. 44:1385-1390

36) Griffin M, Casadio R, Bergamini CM (2002) **Transglutaminases: nature's biological glues**. *Biochem J* 368(Pt 2):377–396

37) Grosso H & Mouradian MM (2012) **Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications.** *Pharmacol Ther.* 133(3):392-410

38) Gundemir S, Colak G, Tucholski J, Johnson GV (2012) **Transglutaminase 2: a molecular Swiss army knife**. *Biochim Biophys Acta* 1823:406–419

39) Hasegawa G, Suwa M, Ichikawa Y, Ohtsuka T, Kumagai S, Kikuchi M, Kikuchi M, Sato Y, Saito Y (2003) **A novel function of tissue-type transglutaminase: protein disulphide isomerase**. *Biochem J* 373(Pt 3):793–803

40) Hynd MR, Scott HL, Dodd PR (2004) **Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease**. *Neurochem. Int*. 45:583–595

41) Ientile R, Caccamo D, Griffin M (2007) **Tissue transglutaminase and the stress response**. *Amino Acids*. 33:385–394

42) Iismaa SE, Mearns BM, Lorand L, Graham RM (2009) **Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders**. *Physiol Rev*. 89(3):991-1023

43) Jakel RJ & Maragos WF (2000) **Neuronal cell death in Huntington's disease: a potential role for dopamine**. *Trends Neurosci.* 23:239–245

44) Jeitner TM, Pinto JT, Krasnikov BF, Horswill M, Cooper AJ (2009) **Transglutaminases and neurodegeneration.** *J Neurochem* (Suppl. 1), 160–166

45) Johnson GV, Cox TM, Lockhart JP, Zinnerman MD, Miller ML, Powers RE (1997) **Transglutaminase activity is increased in Alzheimer's disease brain.** *Brain Res.* 751:323–329

46) Johnson K, Hashimoto S, Lotz M, Pritzker K, Terkeltaub R (2001**) Interleukin-1 induces pro-mineralizing activity of cartilage tissue transglutaminase and factor XIIIa**. *Am. J. Pathol.* 159:149–163

47) Jou MJ (2008) **Pathophysiological and pharmacological implications of mitochondria-targeted reactive oxygen-species generation in astrocyte**s. *Adv Drug Deliv Rev* 60(13–14):1512–1526

48) Junn E, Ronchetti RD, Quezado MM, Kim SY, Mouradian MM. (2003**) Tissue transglutaminase-induced aggregation of alphasynuclein: Implications for Lewy body formation in Parkinson's disease and dementia with Lewy bodies**. *Proc. Natl Acad. Sci.* 100:2047–2052

49) Karpuj MV, Garren H, Slunt H, Price DL, Gusella J, Becher MW, Steinman L (1999) **Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei**. *Proc. Natl Acad. Sci.* 96:7388–7393

50) Kim SY, Grant P, Lee JH, Pant HC, Steinert PM (1999) **Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in Alzheimer's disease**. *J. Biol. Chem*. 274(43):30715-21

51) Király R, Csosz E, Kurtán T, Antus S, Szigeti K, Simon-Vecsei Z, Korponay-Szabó IR, Keresztessy Z, Fésüs L (2009) **Functional significance of five noncanonical Ca2+-binding sites of human transglutaminase 2 characterized by site-directed mutagenesis**. *FEBS Lett*. 276:7083–7096

52) Kuncio GS, Tsyganskaya M, Zhu J, Liu SL, Nagy L, Thomazy V, Davies PJ, Zern MA (1998) **TNF-alpha modulates expression of the tissue transglutaminase gene in liver cells.** *Am. J. Physiol*. 274:G240– G245

53) Lai TS, Hausladen A, Slaughter TF, Eu JP, Stamler JS, Greenberg CS (2001) **Calcium regulates Snitrosylation, denitrosylation, and activity of tissue transglutaminase.** *Biochemistry*. 40:4904–4910

54) Lesort M, Attanavanich K, Zhang J, Johnson GV (1998) **Distinct nuclear localization and activity of tissue transglutaminase**. *Biol Chem* 273:11991–11994

55) Lesort M, Chun W, Johnson GV, Ferrante RJ (1999) **Tissue transglutaminase is increased in Huntington's disease brain.** *J. Neurochem.* 73:2018-2027

56) Lesort M, Tucholski J, Miller ML, Johnson GV (2000) **Tissue transglutaminase: a possible role in neurodegenerative diseases.** *Prog Neurobiol* 61(5):439–463

57) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) **Protein measurement with the Folin phenol reagent**. *J Biol Chem*. 193(1):265-75

58) Lu S, Saydak M, Gentile V, Stein JP, Davies PJ (1995) **Isolation and characterization of the human tissue transglutaminase gene promoter**. *J. Biol. Chem*. 270:9748–9756

59) Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA (2004) **Gene regulation and DNA damage in the ageing human brain**. *Nature* 429:883–891

60) Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M, D'Apolito M, Guido S, Masliah E, Spencer B, Quaratino S, Raia V, Ballabio A, Maiuri L (2010). **Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition**. *Nat Cell Biol* 12(9):863–875

61) Lynch DR & Dawson TM (1994) **Secondary mechanisms in neuronal trauma**. *Curr. Opin. Neurol*. 7:510–516

62) Maccioni RB & Seeds NW (**1986) Transglutaminase and neuronal differenti**ation. *Molec. Cell Biochem*. 69:161-168

63) Mahoney SA, Wilkinson M, Smith S, Haynes LW (2000) **Stabilization of neurites in cerebellar granule cells by transglutaminase activity: identification of midkine and galectin-3 as substrates.** *Neuroscience* 101:141–155

64) Malorni W, Farrace MG, Matarrese P, Tinari A, Ciarlo L, Mousavi-Shafaei P, D'Eletto M, Di Giacomo G, Melino G, Palmieri L, Rodolfo C, Piacentini M (2009) **The adenine nucleotide translocator 1 acts as a type 2 transglutaminase substrate: implications for mitochondrial-dependent apoptosis.** *Cell Death Differ*. 16(11):1480-92

65) Martin A, Romito G, Pepe I, De Vivo G, Merola MR, Limatola A, Gentile V (2006) **Transglutaminase-catalyzed reactions responsible for the pathogenesis of celiac disease and neurodegenerative diseases: from basic biochemistry to clinic.** *Curr Med Chem* 13(16):1895–1902

66) Mastroberardino PG, Iannicola C, Nardacci R, Bernassola F, De Laurenzi V, Melino G, Moreno S, Pavone F, Oliverio S, Fesus L, Piacentini M (2002) **'Tissue' transglutaminase ablation reduces neuronal death and prolongs survival in a mouse model of Huntington's disease.** *Cell Death Differ*. 9:873–880

67) Mastroberardino PG, Farrace MG, Viti I, Pavone F, Fimia GM, Melino G, Rodolfo C, Piacentini M. (2006) **"Tissue" transglutaminase contributes to the formation of disulphide bridges in proteins of mitochondrial respiratory complexes**. *Biochim Biophys Acta*. 1757(9-10):1357-65

68) Mastroberardino PG & Piacentini M (2010) **Type 2 transglutaminase in Huntington's disease: a double-edged sword with clinical potential**. *J Intern Med* 268(5):419–431

69) Mehta K, Fok JY, Mangala LS (2006) **Tissue transglutaminase: from biological glue to cell survival cues**. *Front. Biosci*. 11:173–185

70) Mehta K, Kumar A, Kim HI (2010) **Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer.** *Biochem. Pharmacol*. 80:1921–1929

71) Merz D, Liu R, Johnson K, Terkeltaub R (2003) **IL-8/CXCL8 and growth-related oncogene alpha/CXCL1 induce chondrocyte hypertrophic differentiation**. *J. Immunol*. 171:4406–4415

72) Milakovic T, Tucholski J, McCoy E, Johnson GV (2004) **Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death.** *J. Biol. Chem*. 279:8715–8722

73) Mirza A, Liu SL, Frizell E, Zhu J, Maddukuri S, Martinez J, Davies P, Schwarting R, Norton P, Zern MA (1997) **A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF-kappaB.** *Am. J. Physiol*. 272:G281–G288

74) Mishra S & Murphy LJ (2004) **Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase**. *J Biol Chem* 279(23):23863–23868

75) Mishra S, Melino G, Murphy LJ (2007) **Transglutaminase 2 kinase activity facilitates protein kinase A-induced phosphorylation of retinoblastoma protein**. *J Biol Chem* 282(25):18108–18115

76) Nagy L, Saydak M, Shipley N, Lu S, Basilion JP, Yan ZH, Syka P, Chandraratna RA, Stein JP, Heyman RA, Davies PJ (1996) **Identification and characterization of a versatile retinoid response element (retinoic acid receptor response element-retinoid X receptor response element) in the mouse tissue transglutaminase gene promoter**. *J. Biol. Chem.* 271:4355–4365

77) Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im MJ, Graham RM (1994). **Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function**. *Science* 264(5165):1593–1596

78) Nanda N, Iismaa SE, Owens WA, Husain A, Mackay F, Graham RM (2001) **Targeted inactivation of Gh/tissue transglutaminase II**. *J Biol Chem* 276(23):20673–20678

79) Nardacci R, Lo Iacono O, Ciccosanti F, Falasca L, Addesso M, Amendola A, Antonucci G, Craxì A, Fimia GM, Iadevaia V, Melino G, Ruco L, Tocci G, Ippolito G, Piacentini M (2003**) Transglutaminase type II plays a protective role in hepatic injury**. *Am J Pathol* 162(4):1293–1303

80) Nurminskaya MV & Belkin AM (2012) **Cellular functions of tissue transglutaminase**. *Int Rev Cell Mol Biol.* 294:1-97

81) Ohashi H, Itoh Y, Birckbichler PJ, Takeuchi Y (1995) **Purification and characterization of rat brain transglutaminase.** *J. Biochem*. 118:1271-1278

82) Ozpolat B, Akar U, Mehta K, Lopez-Berestein G (2007) **PKC delta and tissue transglutaminase are novel inhibitors of autophagy in pancreatic cancer cells** *Autophagy* 3(5):480-3

83) Park D, Choi SS, Ha KS (2010) **Transglutaminase 2: a multi-functional protein in multiple subcellular compartments.** *Amino Acids.* 39:619–631

84) Pastuszko A, Wilson DF, Erecinska M (1986) **A role for transglutaminase in neurotransmitter release by rat brain synaptosome**s. *J. Neurochem*. 46:499–508

85) Perry MJ & Haynes LW (**1993) Localization and activity of transglutaminase, a retinoid-inducible protein, in developing rat spinal cord**. *Int. J. devl Neurosci*. 11:325-337

86) Piacentini M, Cerù MP, Dini L, Di Rao M, Piredda L, Thomazy V, Davies PJ, Fesus L (1992a) **In vivo and in vitro induction of 'tissue' transglutaminase in rat hepatocytes by retinoic acid.** *Biochim. Biophys. Acta*. 1135:171–179

87) Piacentini M, Annicchiarico-Petruzzelli M, Oliverio S, Piredda L, Biedler JL, Melino E (1992b) **Phenotypespecific "tissue" transglutaminase regulation in human neuroblastoma cells in response to retinoic acid: correlation with cell death by apoptosis.** *Int. J. Cancer*. 52:271–278

88) Piacentini M, Farrace MG, Piredda L, Matarrese P, Ciccosanti F, Falasca L, Rodolfo C, Giammarioli AM, Verderio E, Griffin M, Malorni W (2002) **Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress**. *J Neurochem* 81:1061–1072

89) Piacentini M, D'Eletto M, Falasca L, Farrace MG, Rodolfo C (2011) **Transglutaminase 2 at the crossroads between cell death and survival**. *Adv Enzymol Relat Areas Mol Biol* 78:197–246

90) Piacentini M, D'Eletto M, Farrace MG, Rodolfo C, Del Nonno F, Ippolito G, Falasca L (2014) **Characterization of distinct sub-cellular location of transglutaminase type II: changes in intracellular distribution in physiological and pathological states** *Cell Tissue Res*. 358(3):793-805

91) Pollack M, Phaneuf S, Dirks A, Leeuwenburgh C Ann (2002) **The role of apoptosis in the normal aging brain, skeletal muscle, and heart.**. *N Y Acad Sci* 959:93-107

92) Porcellotti S, Fanelli F, Fracassi A, Sepe S, Cecconi F, Bernardi C, Cimini A, Cerù MP, Moreno S (2015) **Oxidative Stress during the Progression of β-Amyloid Pathology in the Neocortex of the Tg2576 Mouse Model of Alzheimer's Disease**. *Oxid Med Cell Longev*.

93) Ritter SJ & Davies PJ (1998) **Identification of a transforming growth factor-beta1/bone morphogenetic protein 4 (TGF-beta1/BMP4) response element within the mouse tissue transglutaminase gene promoter**. *J. Biol. Chem*. 273 (21):12798–12806

94) Rodolfo C, Mormone E, Matarrese P, Ciccosanti F, Farrace MG, Garofano E, Piredda L, Fimia GM, Malorni W, Piacentini M (2004) **Tissue transglutaminase is a multifunctional BH3-only protein**. *J Biol Chem* 279:54783–54792

95) Romano AD, Serviddio G, de Matthaeis A, Bellanti F, Vendemiale G (2010) **Oxidative stress and aging** *J Nephrol*. Suppl 15:S29-36.

96) Ruan Q & Johnson GV (2007) **Transglutaminase 2 in neurodegenerative disorders**. *Front Biosci* 12:891–904

97) Salminen A & Kaarniranta K (2009) **Regulation of the aging process by autophagy**. *Trends Mol Med*. 15(5):217-24

98) Sane DC, Kontos JL, Greenberg CS (2007) **Roles of transglutaminases in cardiac and vascular diseases**. *Front Biosci*. 12:2530-45

99) Sarang Z, Molnár P, Németh T, Gomba S, Kardon T, Melino G, Cotecchia S, Fésüs L, Szondy Z (2005) **Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas- mediated cell death in mice.** 42(3): 578–587

100) Sarang Z, Tóth B, Balajthy Z, Köröskényi K, Garabuczi E, Fésüs L, Szondy Z (2009) **Some lessons from the tissue transglutaminase knockout mouse. Some lessons from the tissue transglutaminase knockout mouse**. *Amino Acids*. 36:625–631

101) Selkoe DJ, Abraham C, Ihara Y (1982) **Brain transglutaminase: in vitro crosslinking of human neurofilament proteins into insoluble polymers**. *Proc. natn. Acad. Sci*. 79:6070-6074

102) Sepe S, Nardacci R, Fanelli F, Rosso P, Bernardi C, Cecconi F, Mastroberardino PG, Piacentini M, Moreno S (2014) **Expression of Ambra1 in mouse brain during physiological and Alzheimer type aging**. *Neurobiol Aging.* 35(1):96-108

103) Shen J & Tower J (2009) **Programmed cell death and apoptosis in aging and life span regulation.** *Discov Med*. 8(43):223-226

104) Siegel M & Khosla C (2007**) Transglutaminase 2 inhibitors and their therapeutic role in disease states**. *Pharmacol Ther* 115(2):232–245

105) Siegel M, Xia J, Khosla C (2007) **Structure-based design of alpha-amido aldehyde containing gluten peptide analogues as modulators of HLA-DQ2 and transglutaminase 2.** *Bioorg Med Chem* 15(18):6253–6261

106) Siegel M, Strnad P, Watts RE, Choi K, Jabri B, Omary MB, Khosla C (2008) **Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury**. *PLoS One.* 3(3):e1861

107) Singh US, Erickson JW, Cerione RA (1995) **Identification and biochemical characterization of an 80 kilodalton GTP binding/transglutaminase from rabbit liver nuclei**. *Biochemistry* 34(15):863-871

108) Song H, Kim BK, Chang W, Lim S, Song BW, Cha MJ, Jang Y, Hwang KC (2011) **Tissue transglutaminase 2 promotes apoptosis of rat neonatal cardiomyocytes under oxidative stress**. *J Recept Signal Transduct Res* 31(1):66–74

109) Small K, Feng JF, Lorenz J, Donnelly ET, Yu A, Im MJ, Dorn GW 2nd, Liggett SB (1999**) Cardiac specific overexpression of transglutaminase II (G(h)) results in a unique hypertrophy phenotype independent of phospholipase C activation**. *J Biol Chem* 274(30):21291–21296

110) Stamnaes J, Pinkas DM, Fleckenstein B, Khosla C, Sollid LM (2010) **Redox regulation of transglutaminase 2 activity**. *J. Biol. Chem.* 285:25402–25409

111) Szegezdi E, Macdonald DC, Ni Chonghaile T, Gupta S, Samali A (2009) **Bcl-2 family on guard at the ER.** *Am J Physiol Cell Physiol* 296:C941–C953

112) Szondy Z, Sarang Z, Molnar P, Nemeth T, Piacentini M, Mastroberardino PG, Falasca L, Aeschlimann D, Kovacs J, Kiss I, Szegezdi E, Lakos G, Rajnavolgyi E, Birckbichler PJ, Melino G, Fesus L (2003) **Transglutaminase 2./. mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells**. *Proc Natl Acad Sci* 100(13):7812–7817

113) Szondy Z, Mastroberardino PG, Váradi J, Farrace MG, Nagy N, Bak I, Viti I, Wieckowski MR, Melino G, Rizzuto R, Tósaki A, Fesus L, Piacentini M (2006) **Tissue transglutaminase (TG2) protects cardiomyocytes against ischemia/reperfusion injury by regulating ATP synthesis**. *Cell Death Differ*. 13:1827–1829

114) Takeuchi Y, Birckbichler PJ, Patterson MK Jr, Lee KN (1992) **Putative nucleotide binding sites of guinea pig liver transglutaminase.** *FEBS Lett* 307(2):177–180

115) Thomazy V & Fesus L (1989) **Differential expression of tissue transglutaminase in human cells. An immunohistochemical study.** *Cell Tissue Res* 255(1):215–224

116) Takano K, Shiraiwa K, Moriyama M, Nakamura Y (2010) **Transglutaminase 2 expression induced by lipopolysaccharide stimulation together with NO synthase induction in cultured astrocytes**. *Neurochem Int* 57(7):812–818

117) Tolentino PJ, DeFord SM, Notterpek L, Glenn CC, Pike BR, Wang KK, Hayes RL (2002) **Up-regulation of tissue-type transglutaminase after traumatic brain injury.** *J. Neurochem.* 80:579–588

118) Tolentino PJ, Waghray A, Wang KK, Hayes RL (2004) **Increased expression of tissue-type transglutaminase following middle cerebral artery occlusion in rats.** *J. Neurochem.* 89:1301–1307

119) Tucholski J, Lesort M, Johnson GV (2001) **Tissue transglutaminase is essential for neurite outgrowth in human neuroblastoma SH-SY5Y cells**. *Neuroscience* 102:481–491

120) Tucholski J & Johnson GV (2002) **Tissue transglutaminase differentially modulates apoptosis in a stimulidependent manner.** *J. Neurochem.* 81:780–791

121) Tucholski J, Roth KA, Johnson GV (2006) **Tissue transglutaminase overexpression in the brain potentiates calcium-induced hippocampal damage.***J Neurochem*. 97(2):582-94

122) Verderio EA, Telci D, Okoye A, Melino G, Griffin M (2003) **A novel RGD-independent cel adhesion pathway mediated by fibronectinbound tissue transglutaminase rescues cells from anoikis**. *J Biol Chem* 278:42604–42614

123) Verma A & Mehta K (2007) **Tissue transglutaminase-mediated chemoresistance in cancer cells**. *Drug Resist. Updat.* 10:144–151

124) Villella VR, Esposito S, Bruscia EM, Maiuri MC, Raia V, Kroemer G, Maiuri L. (2013) **Targeting the Intracellular Environment in Cystic Fibrosis: Restoring Autophagy as a Novel Strategy to Circumvent the CFTR Defect**. *Front Pharmacol.* 21;4:1.

125) Vollberg TM, George MD, Nervi C, Jetten AM (1992) **Regulation of type I and type II transglutaminase in normal human bronchial epithelial and lung carcinoma cells**. *Am. J. Respir. Cell Mol. Biol*. 7:10–18

126) Wang JY, Wen LL, Huang YN, Chen YT, Ku MC (2006) **Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation.** *Curr Pharm Des* 12(27):3521–3533

127) Wang Z & Griffin M (2012) **TG2, a novel extracellular protein with multiple func**tions *Amino Acids*. 42(2-3):939-49

128) Zemaitaitis MO, Kim SY, Halverson RA, Troncoso JC, Lee JM, Muma NA (2003) **Transglutaminase activity, protein, and mRNA expression are increased in progressive supranuclear palsy**. *J. Neuropathol. Exp. Neurol*. 62, 173–184

129) Zemskov EA, Janiak A, Hang J, Waghray A, Belkin AM (2006) **The role of tissue transglutaminase in cell-matrix interactions**. *Front Biosci* 11:1057–1076